Targeting oxidative stress for the treatment of COPD and its comorbidities

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

Ivan Keith Gonzales Bernardo

Bachelor of Science
The University of Melbourne

School of Health and Biomedical Sciences
College of Science, Engineering and Health
RMIT University

10 September 2019
Declaration

I certify that the work is that of myself alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Ivan Bernardo
10 September 2019
Thesis Contributions

I would like to thank all of my colleagues that contributed to complete this thesis.

This thesis was conceived and designed by Ivan Bernardo and Professor Ross Vlahos. Ivan Bernardo conducted the animal studies in Chapters 3, 4 and 5. Mr. Huei-Jiunn Seow, Dr. Samantha Passey and Mr. Selcuk Yatmaz assisted Ivan Bernardo with sample collection in Chapters 3 and 4. Dr. Paul Soeding assisted Ivan Bernardo with echocardiographic assessments and Dr. James Wong conducted the strain analysis in Chapter 5.
Acknowledgements

I would like to acknowledge those who have helped and guided me throughout my research. I acknowledge the support I have received for my research through the provision of an RMIT Postgraduate Research Scholarship.

Firstly, I would like to express my appreciation and thanks to my supervisor Prof. Ross Vlahos who has guided me throughout my candidature (and the majority of my adult life). I will always be grateful that you took a chance on the student who walked into your office saying, “I’m not that interested in the lungs.” I wouldn’t be where I am today without the encouragement and support you have given me as a supervisor, mentor and friend.

I would also like to thank my co-supervisors Prof. Steven Bozinovski and Dr. Samantha Passey. Thank you Steve for all your academic input and insight you have provided over the years, especially during those times I felt like I was hitting a brick wall while trying to make sense of my data. Thank you Sam for all that you have taught me, for pushing me when I needed to be pushed, and for checking in during the stressful times.

To the members of the Respiratory Research Group and the wider Chronic Infections and Inflammatory Diseases Program, thank you for making Module D the place to be, especially during those long days where our laughter and banter were the only things that kept us going. Thank you to Huei-Jiunn Seow and Dr. Stanley Chan for teaching me, imparting knowledge and providing technical assistance. Thank you to my peers Amanda Vannitamby and Dr. Simone De Luca for their continuous support throughout this journey as colleagues and as friends. Special thanks to Aleksander Dobric, Kurt
Brassington, Jonathan Erlich, Bashirah Basri, Delphi Kondos-Devcic, Chanelle Mastronardo, Alita Soch and Andrew Chan for keeping morale high during those times when the lab was the last place I wanted to be.

My research would have not have been possible without the aid and support of my family, especially my parents who have supported all my endeavours from day one. Thank you for always believing in me and giving me the opportunity to pave my own way in the world.

Last but not least, I would like to thank my best friend and partner Michelle Bui Hoang. Thank you for your unconditional love and support during these past few years. Thank you for keeping me grounded during the highs and for being my lifeline during the lows. Thank you for sharing this journey with me.
Publications and Presentations

The work in this thesis has given rise to the following publications and conference presentations

**Peer reviewed journal articles**


This work forms the basis of Chapter 1


This work forms the basis of Chapter 5

**Conference abstracts (selected)**


Table of Contents

Declaration .......................................................................................................................... i
Thesis Contributions ......................................................................................................... ii
Acknowledgements ........................................................................................................... iii
Publications and Presentations ......................................................................................... v
Table of Contents ............................................................................................................. vi
List of Tables .................................................................................................................... xii
List of Figures .................................................................................................................. xiii
Common Abbreviations ................................................................................................... xv
Abstract ............................................................................................................................ xvii

Chapter 1: General Introduction ....................................................................................... 1

1. Introduction .................................................................................................................. 2
   1.1. COPD Overview ....................................................................................................... 2
       1.1.1. Acute exacerbations of COPD ......................................................................... 4
   1.2. Oxidative Stress in COPD ...................................................................................... 5
       1.2.1. What is oxidative stress? ................................................................................... 5
       1.2.2. COPD and oxidative stress .............................................................................. 6
       1.2.3. Environmentally-derived ROS ........................................................................ 7
       1.2.4. Cellular derived ROS ...................................................................................... 8
       1.2.5. Reactive nitrogen species ............................................................................... 10
       1.2.6. Depletion of antioxidants ................................................................................. 12
   1.3. Oxidative modifications ......................................................................................... 14
   1.4. Effects of oxidative stress .................................................................................... 16
       1.4.1. Systemic oxidative stress ............................................................................... 17
   1.5. Oxidative stress in comorbidities of COPD ......................................................... 17
   1.6. Skeletal Muscle Wasting ...................................................................................... 18
       1.6.1. Skeletal muscle wasting overview ................................................................. 18
       1.6.2. Mechanisms of skeletal muscle dysfunction ................................................. 20
   1.7. Oxidative stress in skeletal muscle wasting ......................................................... 21
       1.7.1. Current treatments and antioxidant therapies .............................................. 24
1.8. Cardiovascular Disease (CVD) ................................................................. 25
  1.8.1. CVD overview .................................................................................. 25
  1.8.2. CVD and COPD links ...................................................................... 25
  1.8.3. CVD and AECOPD ......................................................................... 26
  1.8.4. Pulmonary hypertension, hypertrophy and heart failure ............... 26
1.9. Oxidative stress in CVD ......................................................................... 27
  1.9.1. Cardiovascular generation of ROS/RNS ........................................ 28
  1.9.2. Oxidative stress in the pathogenesis of CVD ................................. 29
  1.9.3. Nitric oxide in CVD ......................................................................... 30
  1.9.4. Current treatments and antioxidant therapies .............................. 30
1.10. Novel antioxidant approaches for the treatment of COPD ................. 32
  1.10.1. Novel antioxidants ........................................................................ 35
  1.10.2. NOX inhibitors ............................................................................. 36
  1.10.3. Gpx mimetics ................................................................................ 36
1.11. Hypothesis and Aims of this Thesis .................................................... 38

Chapter 2: General Methods, Materials and Experimental Design ............ 41

2. Methods ........................................................................................................ 42
  2.1. In vivo animal studies .......................................................................... 42
    2.1.1. Mice ............................................................................................. 42
    2.1.2. Rats .............................................................................................. 42
  2.2. Experimental procedures ...................................................................... 42
    2.2.1. Cigarette smoke exposure ............................................................ 42
    2.2.2. Chronic cigarette smoke exposure and apocynin groups .............. 43
    2.2.3. Chronic cigarette smoke exposure and ebselen groups ............... 43
    2.2.4. Apocynin preparation and administration .................................... 43
    2.2.5. Ebselen preparation and administration ..................................... 44
  2.3. Sample collection ................................................................................... 44
  2.4. Measures of lung inflammation ............................................................. 44
  2.5. Measures of systemic inflammation and oxidative stress ................. 45
  2.6. Western blotting and oxyblot ................................................................. 45
    Western Blot .......................................................................................... 45
    2.6.1. Tissue homogenization ............................................................... 45
    2.6.2. Protein quantification ................................................................. 45
2.6.3. SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) ................................................. 46
2.6.4. Immunoblotting .................................................................................................................................................. 46
2.6.5. Oxyblot ......................................................................................................................................................... 47
2.7. RNA extraction and RT-qPCR ......................................................................................................................... 48
  2.7.1. Sample crushing ........................................................................................................................................ 48
  2.7.2. RNA Extraction ....................................................................................................................................... 48
  2.7.3. Measurement of RNA yield ..................................................................................................................... 49
  2.7.4. Quantitative polymerase chain reaction ............................................................................................. 49
2.8. Functional analysis of live animals .................................................................................................................... 50
  2.8.1. Muscle Function ....................................................................................................................................... 50
  2.8.2. Transthoracic Echocardiographic Analysis of Ventricular Size and Function ...................................... 51
  2.8.3. Strain Analysis ....................................................................................................................................... 52
  2.8.4. Injection of Agarose Gel and Tissue Collection .................................................................................... 52
  2.8.5. Parameters measured using TTE: ........................................................................................................ 53
2.9. In vitro cell culture studies ............................................................................................................................... 54
  2.9.1. C2C12 mouse skeletal muscle cells ........................................................................................................ 54
  2.9.2. C2C12 storage and handling .................................................................................................................... 54
  2.9.3. General cell culture procedures ............................................................................................................. 55
  2.9.4. Cigarette smoke extract preparation .................................................................................................... 57
  2.9.5. Treatment of C2C12 Cells ......................................................................................................................... 57
  2.9.6. Cell Viability Assays ............................................................................................................................... 58
  2.9.7. MTT Assay .............................................................................................................................................. 58
  2.9.8. MTS Assay ............................................................................................................................................... 60
  2.9.9. Extracellular flux assays ......................................................................................................................... 60
  2.9.10. Treatment of Cells for XF Assay .............................................................................................................. 62
  2.9.11. Seahorse Mito Stress Test ..................................................................................................................... 62
2.10. Statistics ......................................................................................................................................................... 64

Chapter 3: The effect of ebselen in an 8-week cigarette smoke exposure in vivo model of COPD ................................................. 65

3. Introduction ....................................................................................................................................................... 66
  3.1. Materials and Methods .................................................................................................................................. 68
3.1.1. Animals................................................................................................................. 68
3.1.2. Cigarette Smoke Exposure ..................................................................................... 68
3.1.3. Ebselen Administration ......................................................................................... 68
3.1.4. Body Weight and Food Consumption ...................................................................... 69
3.1.5. Muscle Function ..................................................................................................... 69
3.1.6. Blood, BALF and Tissue Collection ...................................................................... 69
3.1.7. Quantitative RT-qPCR ......................................................................................... 70
3.1.8. ELISAs and TBARS .............................................................................................. 70
3.1.9. Oxyblot .................................................................................................................. 71
3.1.10. Statistical Analyses ............................................................................................ 71

3.2. Ebselen Study A ..................................................................................................... 71
3.2.1. Assessment of BALF inflammation ....................................................................... 71
3.2.2. Assessment of cytokine and chemokine mRNA expression in lungs ........ 73
3.2.3. Impact of smoke exposure on total body weight and food consumption ... 75
3.2.4. Impact of cigarette smoke on organ, skeletal muscle and adipose tissue mass ..................................................................................................................... 76
3.2.5. Assessment of hind limb and total limb muscle function ............................ 78

3.3. Ebselen Study B ..................................................................................................... 80
3.3.1. Assessment of BALF inflammation ....................................................................... 80
3.3.2. Impact of smoke exposure on total body weight and food intake .......... 81
3.3.3. Impact of cigarette smoke exposure on organ and skeletal muscle weight .... 83
3.3.4. Assessment of tibialis anterior muscle function in situ ....................................... 85
3.3.5. Assessment of mRNA expression in skeletal muscle tissue .............................. 88
3.3.6. Markers of systemic inflammation and oxidative stress ......................... 89
3.3.7. Assessment of oxidative stress in gastrocnemius tissue ......................... 91

3.4. Discussion .............................................................................................................. 92

Chapter 4: The effect of apocynin in an 8-week CS-exposure in vivo model of COPD .................................................................................................................. 102

4. Introduction .............................................................................................................. 103

4.1. Materials and Methods .......................................................................................... 105
4.1.1. Animals ............................................................................................................... 105
4.1.2. Cigarette Smoke Exposure .................................................................................. 105
4.1.3. Apocynin Administration .................................................................................... 105
4.1.4. Body Weight and Food Consumption .......................................................... 105
4.1.5. Muscle Function ....................................................................................... 106
4.1.6. Blood, BALF and Tissue Collection .......................................................... 106
4.1.7. Quantitative RT-qPCR ............................................................................ 107
4.1.1. ELISAs and TBARS .................................................................................. 107
4.1.2. Oxyblot ..................................................................................................... 108
4.1.3. C2C12 Mouse Skeletal Muscle Cells ......................................................... 108
4.1.4. H₂O₂ and Cigarette Smoke Extract Exposure ......................................... 108
4.1.5. Cell Viability ............................................................................................. 108
4.1.6. Mitochondrial Stress Test .......................................................................... 109
4.1.7. Statistical Analyses ................................................................................... 109
4.2. Apocynin Study A ......................................................................................... 109
4.2.1. Assessment of BALF inflammation ............................................................ 109
4.2.2. Assessment of cytokine mRNA expression in lungs ............................... 111
4.2.3. Impact of smoke exposure on total body weight and food consumption ... 113
4.2.4. Impact of smoke on organ, skeletal muscle and adipose tissue mass ...... 114
4.2.5. Assessment of mRNA expression in tibialis anterior .............................. 117
4.2.6. Assessment of mRNA expression in heart tissue .................................... 119
4.3. Apocynin Study B ......................................................................................... 122
4.3.1. Assessment of BALF inflammation ............................................................ 122
4.3.2. Impact of smoke exposure on total body weight and food intake .......... 123
4.3.3. Impact of smoke exposure on organ and skeletal muscle weights ......... 125
4.3.4. Assessment of tibialis anterior muscle function in situ ....................... 128
4.3.5. Assessment of mRNA expression in skeletal muscle tissue ............... 132
4.3.6. Markers of systemic inflammation and oxidative stress .................... 134
4.3.7. Assessment of oxidative stress in gastrocnemius tissue ..................... 136
4.4. C2C12 In Vitro pilot studies ....................................................................... 138
4.4.1. Impact of H₂O₂ on C2C12 skeletal muscle cell viability ...................... 138
4.4.2. Impact of H₂O₂ on mRNA expression in C2C12 cells ....................... 139
4.4.3. Impact of H₂O₂ on mitochondrial respiration in C2C12 cells ............ 140
4.5. Discussion .................................................................................................... 142

Chapter 5: Evaluation of right heart function in a rat model using modified echocardiographic views .............................................................. 153
5. Introduction .................................................................................................................... 154

5.1. Methods ....................................................................................................................... 155

5.1.1. Echocardiographic Analysis of Ventricular Size and Function......................... 156

5.1.2. Echocardiographic calculations .............................................................................. 158

5.1.3. Strain Analysis ....................................................................................................... 158

5.1.4. Injection of Agarose Gel and Tissue Collection .................................................. 159

5.1.5. Statistics and Analysis .......................................................................................... 160

5.2. Results ......................................................................................................................... 160

5.2.1. Echocardiographic measurement of ventricle size and function ....................... 160

5.2.2. Correlation with necropsy ..................................................................................... 161

5.2.3. Right Ventricular ejection ..................................................................................... 162

5.2.4. 2D-Strain analysis ................................................................................................. 163

5.3. Discussion .................................................................................................................... 165

Chapter 6: General Discussion .......................................................................................... 169

6. Major Findings ............................................................................................................... 170

6.1. Limitations .................................................................................................................. 172

6.2. Future Directions ...................................................................................................... 174

6.2.1. Optimisation of in vivo models to identify the exact role of oxidative stress in skeletal muscle wasting in COPD and validate NOX-2 inhibition .................... 174

6.2.2. Characterisation of the pulmonary manifestations induced by cigarette smoke exposure and the effect of targeted antioxidant therapy ...................... 176

6.2.3. Assessment of targeted antioxidant therapy in animal models of comorbid cardiovascular disease ................................................................. 177

6.3. Conclusion ................................................................................................................... 178

References ......................................................................................................................... 180

Appendices ......................................................................................................................... 204

Appendix 1 .......................................................................................................................... 205
List of Tables

Table 1. Various antioxidant compounds that have been studied in the context of COPD and/or its comorbidities.......................................................................................................................35
Table 2. The effect of ebselen (10mg kg⁻¹) administration on lung, heart, skeletal muscle weights, and WAT weights in mice after cigarette smoke exposure. ......................78
Table 3. The effect of ebselen (10mg kg⁻¹) administration on lung, skeletal muscle weights, and WAT weights in mice after cigarette smoke exposure.................................85
Table 4 The effect of ebselen (10mg kg⁻¹) administration on tibialis anterior tissue mRNA expression of Atrogin-1, MuRF-1, IGF-1, myostatin, IL-6, Gpx-1, Ppargc1a and Nfe2l2 in mice after cigarette smoke exposure.................................................................88
Table 5. The effect of ebselen (10 mg kg⁻¹) administration on gastrocnemius tissue mRNA expression of Atrogin-1, MuRF-1, IGF-1, myostatin, IL-6, Gpx-1, PPARGC1A and Nfe2l2 in mice after smoke exposure..........................................................89
Table 6. The effect of apocynin (5mg kg⁻¹) administration on lung, heart, kidney, liver, spleen, skeletal muscle weights, and WAT weights in mice after smoke exposure. ...........................................................................................................................117
Table 7. The effect of apocynin (5mg kg⁻¹) administration on lung, heart, skeletal muscle, and WAT weights in mice after smoke exposure.......................................................127
Table 8. The effect of apocynin (5mg kg⁻¹) administration on tibialis anterior tissue mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 in mice after cigarette smoke exposure.................................133
Table 9. The effect of apocynin (5mg kg⁻¹) administration on gastrocnemius tissue mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 in mice after cigarette smoke exposure.................................134
Table 10. Cardiac measurement of left and right ventricle chamber size and function....161
Table 11. Taqman assay IDs. ...........................................................................................................205
List of Figures

Figure 1. Overview of oxidative stress in the pathogenesis of COPD. ................................................. 6
Figure 2. Cellular generation of reactive oxygen and nitrogen species in COPD. ................................. 9
Figure 3. The reactive nitrogen species cascade .................................................................................... 11
Figure 4. Oxidants (•OH) abstract the allylic hydrogen from lipid molecules (LH) forming the carbon-centered lipid radical .............................................................................................. 16
Figure 5: Regulation of muscle mass relies on various hypertrophy and atrophy signalling pathways. .................................................................................................................................................. 23
Figure 6. Points of therapeutic intervention in COPD and its comorbidities ........................................... 34
Figure 7. The effect of ebselen (10 mg kg⁻¹) administration on BALF cellularity in mice exposed to air (sham) or after cigarette smoke (smoke) exposure ......................................................... 72
Figure 8. The effect ebselen (10 mg kg⁻¹) administration on pooled lung tissue mRNA expression of inflammatory cytokines and chemokines in mice exposed to air (sham) or cigarette smoke (smoke) exposure ................................................................................................................................. 74
Figure 9. The effect of ebselen (10 mg kg⁻¹) administration on total body weight and food consumption in mice after smoke exposure ............................................................................... 76
Figure 10. The effect of ebselen (10 mg kg⁻¹) administration on forelimb and total limb grip strength in mice after cigarette smoke exposure ...................................................................................... 79
Figure 11. The effect of ebselen (10 mg kg⁻¹) administration on BALF cellularity in mice after smoke exposure ............................................................................................................................................... 81
Figure 12. The effect of ebselen (10 mg kg⁻¹) administration on total body weight and food consumption in mice after smoke exposure ...................................................................................... 83
Figure 13. Effect of ebselen (10 mg kg⁻¹) administration on tibialis anterior contractile function in mice after cigarette smoke exposure ............................................................................................. 87
Figure 14. The effect of ebselen (10 mg kg⁻¹) administration on markers of systemic inflammation and systemic oxidative stress in mice after cigarette smoke exposure ......................................................................................... 87
Figure 15. The effect of ebselen (10 mg kg⁻¹) administration on protein carbonylation in gastrocnemius tissue in mice after smoke exposure .................................................................................. 91
Figure 16. The effect of apocynin (5 mg kg⁻¹) administration on BALF cellularity in mice exposed to air (sham) or cigarette smoke (Smoke) ........................................................................................................... 92
Figure 17. The effect of apocynin (5 mg kg⁻¹) on pooled whole lung tissue mRNA expression of cytokines and chemokines in mice exposed to air (sham) or cigarette smoke (smoke) ................................................................................................. 112
Figure 18. The effect of apocynin administration (5 mg kg⁻¹) on total body weight and food consumption in mice after cigarette smoke exposure. ............................................. 114

Figure 19. The effect of apocynin (5 mg kg⁻¹) administration on pooled tibialis anterior tissue mRNA expression of MuRF-1, atrogin-1, myostatin, IGF-1 and IL-6 in mice after smoke exposure................................................................. 119

Figure 20. The effect of apocynin (5 mg kg⁻¹) administration on heart tissue mRNA expression of NPPA, Myh1, TNF-α, and IL-1α in mice after smoke exposure... 121

Figure 21. The effect of apocynin (5 mg kg⁻¹) administration on BALF cellularity in mice after smoke exposure. ........................................................................... 123

Figure 22. The effect of apocynin (5 mg kg⁻¹) administration on total body weight and food consumption in mice after smoke exposure............................................. 125

Figure 23. The effect of apocynin (5 mg kg⁻¹) administration on tibialis anterior contractile function in mice after smoke exposure......................................................... 131

Figure 24. The effect of apocynin (5 mg kg⁻¹) administration on markers of systemic inflammation and systemic oxidative stress in mice after cigarette smoke exposure.......................................................... 136

Figure 25. The effect of apocynin (5 mg kg⁻¹) administration on protein carbonylation in gastrocnemius tissue from mice after smoke exposure........................................... 137

Figure 26. The effect of increasing H₂O₂ concentrations on C2C12 cell viability at 1 and 3 hours post treatment................................................................. 139

Figure 27. The effect of 10μM of H₂O₂ on C2C12 TNF-α and IL-6 mRNA expression 1 hour post treatment. ............................................................................. 140

Figure 28. The effect of 10 and 30μM H₂O₂ on C2C12 respiration after 1 hour of exposure. ........................................................................................................... 141

Figure 29. Echocardiographic views used to assess cardiac function......................................................... 157

Figure 30. Example of an agarose gel mold of the right ventricle .......................................................... 159

Figure 31. Correlation of RV chamber dimensions with gel mass from necropsy findings ............................................................................................................. 162

Figure 32. Assessment of ventricular function ......................................................................................... 163

Figure 33. Representative 2D-Strain measurement of ventricular contraction........................................ 164
# Common Abbreviations

A list of common abbreviations used across all chapters is provided below. Key words are abbreviated within individual chapters are defined in parentheses where appropriate.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AECOPD</td>
<td>Acute exacerbations of chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>Cat</td>
<td>Catalase</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CS</td>
<td>Cigarette smoke</td>
</tr>
<tr>
<td>CSA</td>
<td>Cigarette smoke exposure and apocynin treatment</td>
</tr>
<tr>
<td>CSE</td>
<td>Cigarette smoke exposure and ebselen treatment / Cigarette smoke extract (in vitro studies only)</td>
</tr>
<tr>
<td>CSV</td>
<td>Cigarette smoke exposure and vehicle treatment</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CXCL-x</td>
<td>C-X-C motif chemokine x</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational force equivalent</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Gpx-x</td>
<td>Glutathione peroxidase-x</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IGF-x</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL-x</td>
<td>Interleukin-x</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>MIP-2α</td>
<td>Macrophage inflammatory protein 2-alpha</td>
</tr>
<tr>
<td>MMP-x</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>Muscle ring factor 1</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>Nuclear Factor, Erythroid 2 Like 2</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOX-x</td>
<td>NADPH oxidase-x</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl</td>
</tr>
<tr>
<td>ONOO</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PPARGC1A</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogren species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>SA</td>
<td>Sham exposure and apocynin treatment</td>
</tr>
<tr>
<td>SC</td>
<td>Modified Scherrer-Crosby</td>
</tr>
<tr>
<td>SE</td>
<td>Sham exposure and ebselen treatment</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SV</td>
<td>Sham exposure and vehicle treatment</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substance</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
</tr>
<tr>
<td>XF</td>
<td>Extracellular flux</td>
</tr>
</tbody>
</table>
Abstract

Chronic obstructive pulmonary disease (COPD) is an incurable global health burden and is characterised by progressive airflow limitation and loss of lung function. In addition to the pulmonary impact of the disease, COPD patients often develop comorbid diseases such as cardiovascular disease and skeletal muscle wasting, lung cancer and osteoporosis. One key feature of COPD, yet often underappreciated, is the contribution of oxidative stress in the onset and development of the disease. Patients experience an increased burden of oxidative stress due to the combined effects of excess reactive oxygen (ROS) and nitrogen species (RNS) generation, antioxidant depletion and reduced antioxidant enzyme activity. Currently, there is a lack of effective treatments for COPD, and an even greater lack of research regarding interventions that treat both COPD and its comorbidities. Due to the involvement of oxidative stress in the pathogenesis of COPD and many of its comorbidities, a unique therapeutic opportunity arises where the treatment of a multitude of diseases may be possible with only one therapeutic target.

In this thesis, we used an established smoke exposure preclinical model of COPD to assess the effectiveness of two novel antioxidant therapies ebeselen and apocynin. Using these compounds, we aimed to determine whether targeting the oxidant-dependent mechanisms that drive COPD and its co-morbidities would have a positive impact on the pulmonary and extrapulmonary manifestations of the disease, specifically in comorbid skeletal muscle wasting. Using this smoke exposure model, we were able to induce several of the key characteristics of COPD including lung inflammation, systemic inflammation, skeletal muscle wasting and oxidative damage.
We assessed the effectiveness of two antioxidant therapies that act on different levels of the reactive oxygen species cascade via differing mechanisms of action. In Chapter 3, we first assessed the efficacy of the Gpx-1 mimetic ebselen, exploring the impact of enhanced hydrogen peroxide reduction in an established animal model of COPD. Ebselen successfully reduced smoke-induced increases in lung inflammation, lung inflammatory cytokine and chemokine mRNA expression. In contrast, Ebselen had no effect on smoke-induced skeletal muscle and WAT wasting, expression of muscle growth signaling pathways, muscle contractility and function.

In Chapter 4, apocynin was used to reduce superoxide production via inhibition of NOX-2 in the same in vivo model of COPD. Apocynin proved to be the more effective compound, as it was able to treat both pulmonary and skeletal muscle manifestations in this model. Apocynin successfully reduced CS-induced increases in lung inflammation, lung inflammatory cytokine and chemokine mRNA expression, and lung mass. Apocynin also reduced smoke-induced skeletal muscle and WAT wasting, improved muscle contractility and function, and reduced oxidative damage to muscle tissue. In addition to this, we explored the use of an in vitro hydrogen peroxide exposure model in C2C12 myotube cells to investigate the mechanisms behind oxidant-dependent alterations in skeletal muscle.

In Chapter 5, we designed, developed and validated a novel methodology of assessing cardiac morphology and function in rodents using transthoracic echocardiography, and aimed to eventually use this methodology to assess the effect of antioxidant therapy in cardiovascular comorbidities of COPD. We demonstrated that examination using the modified mid-right ventricle views has the potential to provide a comprehensive
assessment of the RV particularly when standard transthoracic views are often suboptimal. We found that these views enable a large section of the right ventricle free wall to be imaged, enabling assessment of dilatation, wall hypertrophy and also demonstrated that strain analysis is feasible in rodent models of disease.

Because of the unique interplay between oxidative stress and these diseases, oxidative stress represents a novel target for the treatment of COPD and its comorbidities. The primary findings of this thesis highlight the use of novel antioxidant compounds as viable therapeutic alternatives for the treatment of both pulmonary and extrapulmonary manifestations of COPD. Additionally, validated methods for the identification of the mechanisms that drive the onset and development of comorbidities were established, providing a framework for future studies.
Chapter 1: General Introduction
This general introduction chapter was published as a literature review in the Journal of Pharmacology and Therapeutics in 2015 and entitled “Targeting oxidant-dependent mechanisms for the treatment of COPD and its comorbidities.” It has been adapted for this thesis.

1. Introduction

An increased burden of oxidative stress is an important feature of the pathogenesis of chronic obstructive pulmonary disease (COPD) and its associated comorbid diseases (comorbidities). Current forms of therapy for COPD are largely ineffective and the development of effective treatments for COPD have been severely hampered as the mechanisms and mediators that drive the induction and progression of chronic inflammation, emphysema, altered lung function, defective lung immunity and many extrapulmonary comorbidities are still poorly understood. What is known is that four primary mechanisms have been implicated in the pathophysiological alterations observed in COPD: oxidative stress; inflammation; protease-antiprotease imbalance; and apoptosis [1-3]. Although mostly underappreciated, oxidative stress has been recognized as a central component in the pathogenesis of COPD as it can trigger and further potentiate the other three mechanisms. In this review we focus on the role of oxidative stress in the pathogenesis of COPD and its comorbidities. Given its pivotal role in the onset and development of COPD, oxidative stress may be a novel target for the treatment of COPD and its comorbidities.

1.1. COPD Overview

COPD represents an increasing global burden, afflicting over 600 million people and corresponding to approximately 5% of all deaths globally [4]. It is currently the third leading
cause of death [5]. It is well known that lower/middle income countries bear most of the burden of COPD with almost 90% of COPD-related deaths worldwide taking place in these countries [6]. Although the incidence rate of COPD is likely to escalate in both developed and developing countries, COPD poses a heavier burden on the Asia-pacific and African regions where smoking is still widespread and gradually increasing [7, 8]. Cigarette smoke is inarguably the biggest risk factor for COPD, with 90% of deaths from COPD directly attributable to smoking [9]. Other risk factors include exposure to air pollutants and biomass fuels [10]. In addition, there is generally a long latency period between exposure to smoke and clinically-evident disease and as such, there is a high incidence of COPD in the older population and ex-smokers [11]. This latency period generally lasts a number of years and by the time patients become symptomatic, the damage is already irreversible.

COPD is characterized by persistent airflow limitation and lung inflammation resulting in a progressive decline in lung function [12]. The main symptoms of the disease are chronic cough (smokers cough), excessive mucus production and dyspnoea (particularly during exercise) [11]. In addition to the pulmonary manifestation of COPD, many systemic manifestations occur in the form of comorbid diseases, such as skeletal muscle wasting, cardiac dysfunction, osteoporosis and lung cancer [13-15]. These comorbidities have been associated with increased oxidative stress and are known to affect, or are strong predictors of, the mortality of COPD patients independent of the decline in lung function [16-19].

Although COPD is a largely preventable disease, the numbers of diagnoses continue to increase resulting in ever increasing medical costs to patients, communities and governments [5, 20]. COPD patients generally have long hospital stays, require long-term treatments, and in addition to these medical costs, other “costs” such as an increase in days missed from work and limitations to quality of life, are important consequences of this disease [21, 22].
1.1.1. Acute exacerbations of COPD

Patients with COPD often experience episodes of sudden worsening of symptoms, known as acute exacerbations (AECOPD). The Global initiative for Chronic Obstructive Lung Disease (GOLD) defines an exacerbation as “a change in the patient’s baseline dyspnoea, cough, and/or sputum and beyond normal day-to-day variations, that is acute in onset and may warrant a change in regular medication in a patient with underlying COPD”. These exacerbations are considered to be part of the natural progression and chronicity of the disease and as COPD progresses, exacerbations become increasingly more frequent [23, 24]. Exacerbations result in a dramatic increase in lung inflammation and are associated with increased systemic inflammation compared to stable disease where there is only an increase in oxidative stress markers [25]. This contributes to the worsening of symptoms and it has been shown that following hospitalisation for AECOPD, patients have increased mortality rates [26].

AECOPD is often triggered by respiratory infections, such as those caused by the bacteria *Streptococcus Pneumoniae* and *Influenza A Virus*, or acute exposure to airborne irritants [24, 27]. There are also many non-aetiological risk factors that contribute to the frequency of exacerbations such as age, frequent past exacerbations and the presence of comorbid diseases (especially cardiovascular disease) [28]. The rates of exacerbations vary dramatically depending on the parameters used to define an exacerbation, however, rates of severe AECOPD measured by hospitalisations occur at an approximate rate of 0.5 to 3.6/person-year depending on the study cited [29, 30]. The outcomes of AECOPD can vary from the return to near baseline spirometric parameters to respiratory failure and death. Exacerbations are the largest direct cost for the treatment of COPD due to the length of hospital stays and the frequency of the exacerbations per patient [24, 31, 32].
1.2. Oxidative Stress in COPD

1.2.1. What is oxidative stress?

Oxidative stress refers to the imbalance between the oxidant and antioxidant levels in favour of a pro-oxidant environment in cells and tissues [33]. An oxidant is a species that causes or promotes oxidation and an antioxidant is a molecule that inhibits either the formation of oxidants or inhibits oxidation itself. Oxidative stress arises from the inability of innate antioxidant mechanisms to neutralise oxidants generated endogenously or exogenously resulting in an imbalance between oxidant and antioxidant factors [34]. Consequentially, the oxidants predominate and chronic oxidative stress occurs, leading to the modification of lipids, proteins, and DNA [35, 36]. The harmful modifications caused by oxidative stress are referred to as oxidative damage.

Oxidative stress can result from increased production of oxidants (in the form of free radicals/reactive oxygen and nitrogen species) or from diminished antioxidant levels or reduced antioxidant enzyme activity [33]. The depletion of dietary antioxidants (e.g., vitamins E, C, and D, flavonoids and carotenoids) and micronutrients (e.g., iron, copper, zinc, selenium) can also contribute to oxidative stress. Insufficient amounts impair antioxidant enzyme activity [37]. In chronic inflammatory conditions, such as COPD, oxidative stress primarily results from the increased production of reactive oxygen species (ROS) from exposure to toxins (e.g. cigarette smoke, infection) and continuous activation of endogenous enzymes (e.g. NADPH oxidases) (Figure 1).
The generation of reactive oxidant species both exogenously and endogenously can lead to the onset and development of COPD and its comorbidities. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are found in cigarette smoke but in COPD the primary sources of ROS and RNS are from inflammatory cells including macrophages, neutrophils and epithelial cells. When activated by inhaled irritants (e.g. cigarette smoke), enzymatic (e.g. NADPH oxidase) generation of oxidants leads to the depletion of scavenging antioxidants (e.g. vitamin E), resulting in oxidative stress.

1.2.2. COPD and oxidative stress

The prolonged increase in oxidative stress is a major factor in potentiating both the airway and systemic inflammation in COPD and is known to play a key role in the onset and development of COPD and its comorbidities [38-40]. Direct damage occurs due to the oxidants found in cigarette smoke and from excessive levels of ROS and reactive nitrogen species (RNS) produced as a result of both pulmonary and systemic inflammation. An
increase in ROS production in the airways is reflected by elevated levels of markers of oxidative stress (e.g. superoxide and malondialdehyde) in the airspaces, sputum, breath, lungs, and blood in patients with COPD [25, 35]. These levels of oxidative stress markers are also dramatically increased during exacerbations of COPD [41]. Due to the nature of the oxidative burden and its consequences in the progression of COPD, this review is primarily focused on the role of ROS/RNS in the pathogenesis of COPD and its comorbidities, and the potential pharmacological targets related to ROS signalling.

1.2.3. Environmentally-derived ROS

Each puff of cigarette smoke contains more than $10^{15-17}$ oxidant/free radical molecules and over 4,700 highly reactive chemical compounds, such as aldehydes and quinones, which increases the oxidant burden in smokers [35, 40, 42-45]. The nature of ROS found within cigarette smoke varies from short-lived oxidants, such as the superoxide radical ($O_2^{-}$) and the nitric oxide radical ($NO^-$), to long-lived organic radicals, such as semiquinones that can undergo redox cycling within the epithelial lining fluid of smokers for some considerable period of time [43, 46]. Lung and systemic formation of protein carbonyls in response to cigarette smoke-derived lipid peroxides/carbonyls have also been implicated in the pathogenesis of COPD [38].

In addition to the release of oxidants, cigarette smoking is also associated with an increased amount of myeloperoxidase (MPO) in neutrophils, an oxidising factor that forms hypochlorous acid and converts tyrosine to tyrosyl radical [47]. Studies have shown a correlation between the content of MPO in neutrophils and the degree of pulmonary dysfunction observed in patients [48]. In addition, studies suggest that neutrophil MPO-mediated oxidative stress plays a role in lung inflammation [49]. It is also known that cigarette smoke can cause the activation of alveolar macrophages, which is observed in the
bronchoalveolar lavage fluid (BALF) from the lungs of smokers and COPD patients but not present in non-smokers. The activation of macrophages contributes to the endogenous generation of ROS in the respiratory tract [50, 51].

1.2.4. Cellular derived ROS

Cellular-derived ROS is enzymatically produced by inflammatory and epithelial cells within the lung and/or systemically as part of an inflammatory-immune response towards a pathogen or irritant [52]. Production of ROS by phagocytes can be enhanced by oxidants present in cigarette smoke leading to the release of inflammatory mediators [45]. Several sources for ROS production exist within a cell; however, the primary ROS generator is the enzyme NADPH oxidase (NOX), which comes in various isoforms: NOX-1, NOX-2, and NOX-4 [53, 54]. In humans, NOX-1 and 2 are significant ROS-generators which are made up of an enzyme complex which is present in phagocytic and non-phagocytic cells including epithelial cells, macrophages, and skeletal muscles [55, 56]. Although NOX is found in many cell types, it is latent in neutrophils under normal circumstances [57]. Once activated, neutrophils and macrophages can generate ROS via the NADPH oxidase system, leading to further augmentation of oxidative stress in the lungs of smokers and COPD patients (Figure 2).
Figure 2. Cellular generation of reactive oxygen and nitrogen species in COPD.

Cigarette smoke acts on inflammatory cells in the lung (e.g. macrophages, neutrophils, epithelium) where activation of NADPH oxidase 2 (Nox2) generates superoxide radicals ($O_2^{-}$) which can then either react with nitric oxide ($NO$) to form the reactive peroxynitrite molecule ($ONOO^{-}$) or be rapidly converted to hydrogen peroxide ($H_2O_2$) via the enzymatic activity of superoxide dismutase (SOD). In the presence of $Fe^{2+}$, $H_2O_2$ can be converted into the more damaging hydroxyl radical (‘OH) via the Fenton reaction. This reaction causes the oxidation of $Fe^{2+}$ to $Fe^{3+}$, and in this oxidation state, the presence of iron can directly generate ‘OH from $O_2^-$. These iron reactions have increased importance in COPD as a higher concentration of iron has been reported in the lungs of smokers, thereby increasing the potential ROS burden [58]. The glutathione peroxidase (Gpx) family of enzymes, and catalase (CAT) are responsible for the conversion of $H_2O_2$ into harmless water and oxygen, which effectively reduces circulating ROS and thus reduces the oxidative burden [59].

NOX-generated ROS have long been recognized to play key roles in the pathogenesis of a number of diverse chronic lung disorders that result in obstructive physiology, in particular asthma, cystic fibrosis, and emphysema [56]. Mice deficient in p47phox or NOX-2 exhibit increased cigarette smoke–induced lung inflammation and emphysema despite decreased ROS production compared with control mice [60]. The lung responses in p47phox- and NOX2-null mice were associated with increased production of pro-inflammatory
cytokines and chemokines via a TLR4-NF-κB pathway, indicating that NOX-2 may mediate anti-inflammatory functions by restraining TLR4 activation [60]. However, another group reported that p47phox-null mice have less inflammation, IL-6, keratinocyte-derived chemokine, and monocyte chemoattractant protein-1 in lung-lavage specimens after cigarette-smoke exposure compared with WT mice [61]. The differences observed by these groups may be due to variability in lung compartment sampling, cellular distributions, and chronicity of cigarette-smoke exposure.

1.2.5. Reactive nitrogen species

Although increased production of ROS is the primary mechanism of oxidative stress in COPD/chronic lung diseases/acute lung diseases, there is compelling evidence to suggest that RNS also play a role in disease [62, 63]. Reactive nitrogen species include nitric oxide (NO\(^\cdot\)), a nitrogen free radical, and its derivative species such as peroxynitrite and nitrogen dioxide (Figure 3). As with ROS, in addition to generation endogenously, RNS are present in cigarette smoke and air pollutants in the form of NO\(^\cdot\) and has many of the same harmful effects as ROS [64]. Endogenously, NO\(^\cdot\) is associated with a multitude of signalling pathways in mammalian physiological and pathological processes; however, in excess it too causes indiscriminate damage to surrounding tissues and can react with \(O_2^-\) forming the even more harmful peroxynitrite radical (ONOO\(^\cdot\)).
In addition to its ability to cause direct damage, ONOO\(^{-}\) can also react with carbon dioxide (CO\(_2\)) \textit{in vivo}. The reaction between ONOO\(^{-}\) and CO\(_2\) occurs rapidly under physiological conditions, forming nitrosoperoxycarbonate (ONOOCO\(_2\)^\(-\)). ONOOCO\(_2\)^\(-\) homolyzes (the molecule dissociates into 2 free radicals) to form the carbonate radical (\(\cdot\)CO\(_3\)^\(^{-}\)) and nitrogen dioxide (\(\cdot\)NO\(_2\)) \([33]\). It is these radicals (\(\cdot\)CO\(_3\)^\(^{-}\) and \(\cdot\)NO\(_2\)) that are believed to cause peroxynitrite-related cellular damage. The conjugate acid of peroxynitrite, peroxynitrous acid (HNO\(_3\)) also homolyzes into \(\cdot\)OH and \(\cdot\)NO\(_2\) radicals, adding to the oxidative burden caused by peroxynitrite \([65]\).

\textbf{Nitric oxide}

For years, NO\(^{-}\) had only been considered as a toxic, unstable free radical gas that was just a constituent of air pollutant and cigarette smoke. However, it is now known that NO\(^{-}\) can be generated endogenously in several types of cells \([66]\). This implicated NO\(^{-}\) in various physiological roles and pathways, including host defense, vascular regulation and neuronal communication \([33]\). NO\(^{-}\) has been well characterized in human biology and is perhaps the most important endogenous vasoprotective molecule in addition to its role in cardiovascular function \([67-70]\). NO\(^{-}\) inhibits vascular smooth muscle contraction and growth, platelet
aggregation, and leukocyte adhesion to the endothelium, contributing to vascular homeostasis [67-72]. Individuals with hypertension, atherosclerosis and/or diabetes often show impaired NO’ signalling, highlighting the importance of NO’ with regards to comorbid cardiovascular disease [73] (see below).

Nitric oxide is generated by phagocytes and is biosynthesized endogenously from the amino acid L-arginine, oxygen, and NADPH by various nitric oxide synthase (NOS) enzymes [74]. In addition to its signalling roles, NO’ has both pro-oxidant and antioxidant activities; excessive NO’ can cause direct oxidative damage, however, NO’ can also scavenge circulating ROS [75, 76]. More importantly, O$_2^{•-}$ can react directly with endothelium-derived NO’ forming the harmful peroxynitrite (ONOO’) molecule.

**Peroxynitrite**

Peroxynitrite is formed by the reaction between O$_2^{•-}$ and NO’ in vivo. The pairing of these 2 radicals results in ONOO’ anion, which is not a free radical but is still a very potent oxidant. ONOO’ is both an oxidizing and nitrating agent and can thus damage a wide array of molecules, including DNA and proteins [77, 78]. The formation of ONOO’ is of particular relevance to the comorbidities of COPD as its formation involves the consumption of NO’, reducing the bioavailability of NO’ for physiological processes (see Section 3.2.3).

**1.2.6. Depletion of antioxidants**

ROS/RNS generated exogenously or endogenously, whether circulating or in pulmonary vasculature, are scavenged by blood antioxidants and antioxidant enzymes. Accordingly, the ability to protect against the deleterious effects of oxidative stress depends greatly on the antioxidant capacity of the blood and the tissues [79, 80]. Studies have shown that in addition to the increased levels of circulating oxidants, there is also an observed decrease in systemic
antioxidant capacity in smokers and patients with COPD [81]. This is due to the saturation of lung antioxidants, plasma antioxidants and protein sulphydryls by the excessive amounts of circulating ROS released by neutrophils and macrophages [81]. The saturation of many of these antioxidants, such as uric acid, glutathione (GSH), vitamin E, and ascorbate is also associated with the severity of COPD exacerbations [82]. Cigarette smoke, the main etiological risk factor for COPD, has also been shown to irreversibly modify glutathione to glutathione conjugates in the airway epithelium resulting in antioxidant deficiency and injurious lung response [83]. Cigarette smoking also inhibits the protective expression of the Nrf2/antioxidant response element pathway in peripheral mononuclear cells of smokers, favouring a pro-inflammatory state [84]. In addition to the antioxidant saturation, studies have also shown a decrease in anti-oxidative enzyme function in COPD patients; specifically reduced SOD and Gpx activity [85].

There is evidence to suggest that the anti-oxidant enzyme glutathione peroxidase-1 (Gpx-1) may have a role in regulating the inflammatory response to cigarette smoke exposure. Elevated levels of H$_2$O$_2$ are measured in the exhaled breath condensate of COPD patients, particularly during exacerbations [86]. There is upregulation of Gpx-1 gene expression in the lungs of smokers [87] and depletion of Gpx activity in COPD patients and smokers [88-90]. With respect to reduced Gpx activity in COPD patients and smokers, erythrocyte Gpx activity was significantly lower in patients with severe COPD compared with patients with moderate COPD and there is a direct relationship between systemic Gpx activity and [90]. In addition, Gpx activity was decreased in plasma from COPD patients and oxidative stress correlates with both lung function and body mass index in COPD [88]. Moreover, Gpx activity was decreased in total blood from smokers and ex-smokers [89]. However, these studies did not identify the isoform of Gpx that was involved in reduced activity of Gpx.
1.3. Oxidative modifications

Oxidative stress causes a wide array of physiological and pathological consequences not necessarily limited to just COPD patients. In COPD, increased oxidative stress can cause cell damage, cell necrosis, apoptosis, autophagy, remodelling of extracellular matrix and blood vessels, endothelial dysfunction, inactivation of antiproteases, premature cellular senescence, elevated mucus secretion, steroid resistance, unfolded protein response, cell proliferation, epigenetic changes, and autoimmunity [35, 40, 91]. Oxidative modifications of DNA, proteins and lipids all contribute to the pathophysiology of the disease.

**DNA**

Unlike the other ROS and RNS which do not react with DNA bases or deoxyribose, the hydroxyl radical and peroxynitrite can react with both purine (adenine & guanine) and pyrimidine (cytosine & thymine), forming specific products [92]. This oxidative damage can lead to mutation of DNA bases caused by AT-GC transition and GC-TA transversion and, if left unrepaired, can result in changes in protein gene expression [33]. In addition to this, ROS/RNS-induced DNA damage also involves single- or double-stranded DNA breaks and DNA cross-links, DNA damage inducing either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability [93].

**Protein**

Oxidative modifications to proteins caused by ROS/RNS include protein fragmentation, oxidation of amino acids, the formation of carbonyls, dityrosine and nitrated and chlorinated tyrosines [94, 95]. In COPD and other inflammatory diseases, elevated levels of nitrated, chlorinated and brominated tyrosines have been detected in the tissues of patients [96]. These oxidative changes to proteins can result in various functional consequences, such as
inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, modifications in uptake by cells, and altered immunogenicity [97].

**Lipids**

In the presence of ROS/RNS and oxygen, lipids undergo a chain oxidation reaction leading to peroxidation. This chain reaction is initiated by oxidants, primarily the hydroxyl radical and leads to the formation of lipid peroxide and lipid radicals, which can then reinitiate the chain reaction [98]. This process continues indefinitely unless terminated by phenolic antioxidants, also known as chain-breaking antioxidants (e.g. vitamin E) [33] (Figure 4). This is especially important in comorbid cardiovascular disease where the formation of plaques in the arteries is initiated from lipid peroxidation [99].
Figure 4. Oxidants (‘OH) abstract the allylic hydrogen from lipid molecules (LH) forming the carbon-centered lipid radical.

This radical then undergoes molecular rearrangement to form a more stable form of the carbon-centered radical (L’). In the presence of oxygen, L’ rapidly forms a lipid per oxy radical (LOO’). This new radical can then abstract an allylic hydrogen from another lipid molecule generating a new L’. This process can be terminated by the donation of a hydrogen atom from vitamin E to the LOO’ species forming a vitamin E radical. The vitamin E radical then reacts with another LOO’ forming non-radical products and thus terminating the reaction.

1.4. Effects of oxidative stress

ROS and RNS normally play roles in cell signalling and homeostasis; however, in excess they become increasingly toxic. The ROS and RNS O2•−, ONOO−, H2O2 and ¢OH, in addition to their indiscriminate oxidization of proteins, lipids and DNA [100], can result in direct lung damage or induce an assortment of cellular responses. Additionally, these oxidants also influence the inflammatory responses in the lungs via various pathways such as the activation
of transcription factor NF-κB, and transcription of pro-inflammatory mediator genes, further potentiating the inflammatory response in COPD [58]. This induces a pro-inflammatory state that drives the sustained production of more ROS, leading to a vicious cycle of oxidative stress and inflammation. The oxidative damage and modifications caused by ROS are known to precede the characteristic respiratory changes seen in COPD such as airway remodelling and emphysema [101]. Recent studies have also shown a decrease in glucocorticosteroid receptor expression as a result of prolonged oxidative stress indicating that in a pro-oxidant environment, such as in COPD patients, steroid therapy is of limited therapeutic benefit highlighting the need for new treatments [102].

1.4.1. Systemic oxidative stress

Even after smoke cessation, systemic oxidative stress still persists in COPD patients [103]. This is reflected by an elevation in circulating ROS and a depletion of antioxidants [104]. The increased oxidative stress is likely due to the persistent low grade systemic inflammation resulting in the production of ROS systemically [105]. This systemic oxidative stress has been implicated in various systemic manifestations of COPD, with many studies associating this increased burden of oxidative stress with skeletal muscle wasting and cardiovascular disease in COPD [105-107]. In addition to the links between oxidative stress in COPD and comorbid diseases, increased extrapulmonary oxidative stress is also an independent risk factor of many comorbidities such as cardiovascular disease, osteoporosis and depressive disorders in patients without COPD [108-110].

1.5. Oxidative stress in comorbidities of COPD

A prominent feature of COPD is the presence of comorbid diseases (or comorbidities) in patients [13, 15]. These diseases can range from more “mild” or indirectly fatal conditions
that affect quality of life such as osteoporosis and depression, to more directly fatal diseases such as ischaemic heart disease and cancer. COPD-related mortality is likely underestimated due to these comorbidities as their presence makes it difficult to determine the precise cause of death. Respiratory failure is considered to be the major cause of death in advanced COPD; however, comorbidities such as cardiovascular disease and lung cancer are also major causes of death. In early/mid stages of COPD, comorbidities are the leading causes of mortality [19]. Comprehensive studies on the specific cause of deaths in COPD patients have found that rather than dying from progressive respiratory failure, many COPD patients die from a complex web of interconnected comorbidities [102]. For the purpose of this review, comorbidities are defined as the presence of one or more distinct diseases or disorders that are not directly related to COPD or are not part of the natural history/progression of the disease (e.g. respiratory infections resulting in AECOPD). The underlying mechanisms resulting in these systemic manifestations in COPD are not fully understood; however, they have been linked to the persistent low-grade systemic inflammation and persistent oxidative stress in COPD as discussed earlier.

1.6. Skeletal Muscle Wasting

1.6.1. Skeletal muscle wasting overview

Skeletal muscle wasting, also referred to as cachexia or skeletal muscle atrophy, occurs in approximately 20 to 40% of all COPD patients [17, 111, 112]. Muscle wasting is characterized by a marked decrease in skeletal muscle mass, an increase in proportion of type I muscle fibres in the diaphragm and type 2 muscle fibres in the periphery, and associated with low exercise capacity and skeletal muscle weakness [112]. Skeletal muscle wasting in COPD is a strong predictor of mortality, independent of decline in lung function [16-18]. Although present in a large population of patients, the prevalence of muscle wasting can only be approximated as there are no simple techniques to measure muscle mass. Knowing this,
the actual prevalence and extent of muscle wasting in the COPD population is likely underestimated. This is because the data is extrapolated from body weight measurements and lean body mass, an index of muscle mass, may be reduced despite the preservation of total body weight. This is further supported by the fact that patients have a proportionally greater reduction in thigh muscle cross-sectional area compared to reduction in body weight [16]. This indicates that a preferential loss of muscle tissue exists in emaciated patients with COPD [96].

Skeletal muscle wasting, although not a directly fatal condition, reduces health-related quality of life and decreases survivability for COPD patients. The main feature of comorbid skeletal muscle wasting is a reduction in fat free mass (FFM) and is associated with weaker peripheral muscles, impaired functional status, as well as poor health-related quality of life [113, 114]. In COPD, the reduction in muscle mass is proportional to that of the reduction in strength indicating that the remaining contractile apparatus may be functionally preserved. However, in patients chronically treated with glucocorticosteroids, it is possible for the loss of strength to be disproportional to the reduction in muscle mass.

Deteriorations in FFM have also been described following acute exacerbations. These COPD patients also experience a reduction in exercise capabilities, with skeletal muscle alterations contributing to limitations in exercise in addition to pulmonary dysfunction [115]. It should be noted that the strength of the quadriceps is a key determinant of exercise tolerance in COPD. This is explained by the influence that muscle strength has on the perceived leg effort required during exercise, which is considered to be the main limiting symptom in 40–45% of patients with COPD [116].
1.6.2. Mechanisms of skeletal muscle dysfunction

The pathophysiological interaction between COPD and alterations in skeletal muscle tissue is poorly understood and represents an important gap in knowledge of the disease. Skeletal muscle wasting in COPD is multifactorial in nature with several of these factors likely interacting. Many factors (e.g. inflammation, oxidative stress, and poor nutrition) can initiate or enhance alterations in skeletal muscle, such as change in fibre type phenotypic expression and regenerative defects in peripheral muscles of patients with COPD [112]. In this review, multiple risk factors will be briefly discussed along with a more in depth discussion regarding the role of oxidative stress and ROS.

**Smoking**

Cigarette smoking or exposure to other airborne irritants is unlikely to be the primary mechanism involved in skeletal muscle wasting in COPD, as seen in several studies where patients and control subjects were matched for smoking history [112]. Nevertheless, smoking does have some effect on muscle biology and it may predispose patients to the development of skeletal muscle dysfunction [117, 118]. Smoking is also associated with skeletal muscle weakness in otherwise healthy individuals [119-121].

**Disuse**

Peripheral (or limb) muscle dysfunction in COPD has been partly attributed to a reduction in physical activity, or “deconditioning.” In general, the disuse of muscle can lead to many of the features and alterations of skeletal muscle in COPD patients: muscle weakness, muscle atrophy, loss of type I fibres, decreased cross-sectional area of muscle fibres, reduced oxidative enzyme activity, reduced capillary-to-fibre ratio, early lactate release, reduced rate of phosphocreatine synthesis after exercise and altered redox status [122-125]. In healthy
adults these changes are fully reversible in response to training and increased exercise; however, full recovery is unusual in COPD patients [125-128].

Inflammation

A low body mass index (BMI) has been linked to systemic inflammation in COPD patients. As seen in Figure 5, inflammation plays a key role in the activation of protein breakdown in skeletal muscle. Inflammation results in the production of key cytokines, such as IL-8, that can generate an array of cellular responses. This includes the induction of the ubiquitin proteasome (UbP) system through the transcriptional activities of NK-κB and FOXOs, apoptosis, and macroautophagy, all of which have been linked to the muscle atrophy [129]. In COPD patients, there is a lack of evidence with regards to inflammation in skeletal muscle during stable disease [130, 131]; however, increased inflammation is seen during periods of exacerbation [132, 133]. Because of this, the role of inflammation as the key event for the development of skeletal muscle dysfunction in COPD is still widely debated.

Hypoxia

In humans [134] and animals [135] muscle mass decreases under hypoxic conditions. COPD patients that have low arterial O₂ and reduced O₂ delivery tend to have lower body mass than those with normal levels of arterial O₂ and sufficient O₂ delivery [136]. Hypoxia may be a factor driving changes in limb muscle tissue as hypoxia can induce downstream effects leading to the activation of the UbP system and reduced myogenesis [137].

1.7. Oxidative stress in skeletal muscle wasting

In addition to chronic inflammation, hypoxia, cigarette smoke, sepsis and an increased cost of breathing cause the increased generation of oxidants in the lungs (e.g. H₂O₂, O₂⁻, MDA) [35].
It is suggested that, in addition to inflammatory mediators, these oxidants can spill into circulation, increasing the systemic oxidative stress burden of COPD patients [138]. In *in vivo* models of skeletal muscle wasting in COPD, increased oxidative stress has been observed [118]. This increase in oxidative stress can modify muscle proteins, reducing their integrity and enhancing their degradation [139]. Direct exposure to oxidative stress from environmentally derived oxidants, or indirect exposure via cellular-derived ROS due to inflammation, can induce proteolysis. Acute bouts of physical exercise and acute exacerbations can also increase the level of oxidative stress [25, 140, 141]. It is suggested that oxidative stress may acutely affect skeletal muscle function by inhibiting the activity of the sodium/potassium pump, sarcoplasmic reticulum function, myosin ATPase and mitochondrial respiration [115]. In addition to these acute effects, chronic oxidative stress also contributes to muscle wasting and dysfunction in both respiratory and peripheral muscle. The increased presence of ROS associated with COPD causes an increase in the expression of UbP components leading to increased protein breakdown in skeletal muscle (Figure 5) [112].
Figure 5: Regulation of muscle mass relies on various hypertrophy and atrophy signalling pathways.

In skeletal muscle wasting, there is an imbalance between these anabolic and catabolic processes resulting in enhanced muscle degradation as a result of protein breakdown. Myostatin (a negative regulator of muscle mass) can inhibit muscle growth. Inflammatory mediators and ROS can lead to the downstream activation of atrophy-related genes (e.g. Atrogin-1, MuRF-1) resulting in enhanced protein degradation. Inflammation and oxidative stress have also been implicated in the activation of the ubiquitin-proteasome (UbP) system. The UbP system is the primary mechanism of the protein catabolism in mammalian skeletal muscle. Refer to Maltais et al. 2014 for an exhaustive overview of the signalling pathways involved.

There is evidence that COPD patients with muscle wasting experience a more severe abnormal oxidative stress response to submaximal and maximal exercise compared to non-muscle-wasted patients with COPD [142]. Although most studies focus on the increased
signalling of atrophy pathways as the major driving force of comorbid skeletal muscle wasting, it should be noted that decreases in hypertrophy signalling may also contribute greatly to the pathology of the disease. Further studies are required to determine the exact contribution of these various pathways to the disease.

1.7.1. Current treatments and antioxidant therapies

There are currently no effective drug treatments for skeletal muscle wasting in COPD [112]. Pharmacological treatments that have been explored include, anabolic steroids, growth hormone, other growth anabolic compounds and bioactive nutrients (e.g. ghrelin, creatine) and antioxidants [143-145]. Most of these treatments have only shown little to modest benefits when treating skeletal muscle wasting and weakness in COPD, or have significant adverse effects. As with CVD, skeletal muscle wasting and COPD also overlap with regards to their non-pharmacological management. Currently, the most potent treatment for limb muscle dysfunction is exercise training, which is already a key component for the management of COPD [112]. Some treatments such as lifestyle modifications (diet and exercise) and anabolic steroids have shown improvement in patients; however, they do not reverse the progression of muscle loss and may only increase muscle growth without substantial improvements in strength or endurance [146, 147]. Therefore, new treatments are needed as limiting muscle wasting in COPD will lead to improved quality of life and increased survivability of patients. Because of its role in the pathogenesis of comorbid skeletal muscle wasting, targeting oxidative stress may be able to slow the progression or onset of the disease, giving patients an improved quality of life while managing their other symptoms. Furthermore, it has been reported that increasing antioxidant potential can improve muscle performance whilst attenuating muscle fatigue [112]. Traditional antioxidant therapy using scavenging molecules has shown limited improvements in preclinical murine
models of skeletal muscle wasting induced by cigarette smoke. Further pre-clinical and clinical studies are required to determine the effectiveness of targeting oxidative stress in comorbid skeletal muscle wasting.

1.8. Cardiovascular Disease (CVD)

1.8.1. CVD overview

CVD is a broad term used to describe any disease involving the heart and/or blood vessels. The presence of CVD is very common in COPD patients and is the leading cause of morbidity and mortality in younger patients and patients with mild-to-moderate COPD [19]. Comorbid CVD can manifest itself in one or more various disorders such as angina, stroke, arrhythmias, hypertrophy of the heart, and myocardial infarction, and its presence greatly reduces the survivability of COPD patients [107].

1.8.2. CVD and COPD links

It is well documented that COPD and CVD share many of the same risk factors, such as smoking, poor diet, and pre-existing hypertension. When considering these risk factors it is of significance that many, if not all, contribute to disease progression at least in part via oxidative stress. Multiple studies have shown links between COPD and classical cardiovascular risk factors such as family history of coronary heart disease and diabetes [148-151]. Persistent low-grade systemic inflammation is present in both COPD and CVD and oxidative stress, which plays a major role in COPD, has also been implicated in CVD [106]. This indicates that the systemic inflammation and increased oxidative stress in COPD may lead to the onset and development of comorbid CVD.

In addition to this, acute inflammatory lung conditions, such as bacterial/viral infection or acute exposure to airborne irritants, are also associated with vascular dysfunction [152, 153]. Increased systemic inflammation, as a result of these lung conditions, is capable
of destabilizing vulnerable plaques, inducing a prothrombotic state [154]. COPD patients generally do not tolerate cardiac injury or intervention as well as healthy individuals. COPD patients with acute myocardial infarction have a five-year survival rate of 46% compared to 68% in those without COPD [149].

1.8.3. CVD and AECOPD
Although many factors have been associated with poor outcomes from AECOPD, CVD is becoming increasingly recognized as a strong predictor of in-hospital mortality. Studies have shown that over 50% of patients hospitalized for AECOPD have a high prevalence of coexisting cardiovascular disease [155]. Comorbid cardiovascular disease is independently associated with increased risk of AECOPD [156]. The severity of airway obstruction is a major predictor of AECOPD, and is also an independent risk factor for cardiovascular disease [26, 157-159]. There is also mounting evidence associating a high frequency of acute CVD with acute respiratory illness, such as pneumonia or AECOPD. Studies have shown that in the general population, subjects with respiratory tract infections are more likely to experience a myocardial infarction within 2 weeks of infection [160, 161]. A retrospective review examining 24 hour mortality following AECOPD hospitalisation found that approximately 60% of deaths that occurred resulted from cardiovascular causes [162].

1.8.4. Pulmonary hypertension, hypertrophy and heart failure
There are two principal pathological features in the pulmonary vasculature common to most forms of pulmonary hypertension: excessive vasoconstriction and remodelling of the pulmonary arteriolar wall, which primarily occurs by a mechanism of smooth muscle proliferation within the medial layer [163]. Because ROS may promote vasoconstriction,
smooth muscle cell proliferation, and vascular remodelling, oxidative stress likely plays a critical role in many forms of pulmonary hypertension.

Multiple studies have shown that 25-70% of COPD patients have pulmonary hypertension, depending on the definition used [164, 165]. In addition to this, it is estimated that 25% of patients with moderate-to-severe COPD develop pulmonary hypertension within 6 years if they have no hypertension at baseline [166]. The pathological changes implicated in the development of pulmonary hypertension can also be seen in tissue samples of COPD patients who do not have a diagnosis of pulmonary hypertension [167, 168].

A major feature of COPD is emphysema, contributing to airway obstruction. The destruction of alveolar walls and enlargement of airspaces results in a reduction in gas exchange [83]. To compensate for this, blood pressure is increased to facilitate more pulmonary blood flow leading to pulmonary hypertension. This results in increased work in right heart and over time this increased workload can lead to concentric hypertrophy of the right ventricle (RV) [169]. This can lead to reduced ejection fraction, elevated end diastolic RV pressure, impedance of the right heart and, if left unchecked, can lead to heart failure. These changes can be detected in COPD patients, especially in end-stage COPD patients, using transthoracic echocardiography, which allows for the measurement of various parameters in the heart such as systolic and diastolic wall thickness, and chamber size. Both pulmonary hypertension and right heart failure are associated with an increase in morbidity and mortality in COPD patients, independent of the decline in lung function [170].

1.9. Oxidative stress in CVD

The presence of oxidative stress in the form of increased ROS/RNS formation (e.g. $O_2^-$, ONOO⁻) has been observed both clinically and in animal models of CVD [171, 172]. Although implicated in CVD, the cause-effect relationship of oxidative stress with any of the
different cardiovascular diseases has yet to be established. The increased generation of ROS due to impaired mitochondrial reduction of molecular oxygen, secretion of ROS by inflammatory cells, endothelial dysfunction, auto-oxidation of catecholamines, as well as exposure to radiation or air pollution can drive the oxidative stress in cardiac and vascular myocytes [173]. In addition to the increased oxidant production, depression in the antioxidant reserve has also been implicated in CVD. These typically phenolic antioxidants (e.g. vitamin E) act as a protective mechanism in cardiac and vascular myocytes, and their reduced levels appear to be due to both the saturation of by excess ROS/RNS and/or changes in gene expression. The harmful effects caused by ROS/RNS in cardiovascular tissues are mainly due to ability of the oxidant species to drive changes in subcellular organelles (e.g. mitochondria), reduce NO’ bioavailability, and induce intracellular Ca\(^{2+}\)-overload [173].

1.9.1. Cardiovascular generation of ROS/RNS

As stated earlier, in addition to phagocytising cells, NOX-dependent ROS-generation has been observed in numerous non-phagocytising cells, though at a lower level. In the cardiovascular system these include vascular smooth muscle cells (VSMC), endothelial cells, adventitial and cardiac fibroblasts and cardiomyocytes [174]. Normally, these cells continuously generate low levels of ROS even in the absence of external stimuli, and ROS derived from vascular NOX act as second messengers in VSMC signalling. Over long periods of time, vascular NOX complexes only produce low levels of \(\text{O}_2^-\), with much of it generated intracellularly where it participates in cell signalling (compared to phagocyte \(\text{O}_2^-\) which is generated extracellularly) [175]. When stimulated however, there is a significant increase in NOX-driven ROS production by these cells. It is important to recognise that both vascular and phagocytic NOX play an important role in superoxide production as phagocytes
can infiltrate cardiovascular tissues and facilitate the functional and structural alterations observed in CVD [176].

1.9.2. Oxidative stress in the pathogenesis of CVD

Damage to the endothelium is the initiating step in CVD. This damage can expose endothelial cells, along with the underlying cell layers, to the deleterious effects of the inflammatory process, which can ultimately lead to the formation of atherosclerotic lesions. Cellular oxidative stress caused by excess ROS/RNS production is considered to be intrinsic to atherosclerotic lesion formation [177]. Exogenous factors contributing to oxidative stress such as smoking and comorbid diabetes also contribute to vascular oxidative stress and are strong risk factors for CVD [178]. Cigarette smoke has been associated with the down-regulation of key exogenous and endogenous antioxidants such as vitamin C (ascorbic acid), carotene, Gpx and SOD [179, 180]. This can lead to dysfunction in endothelial cells, monocytes and VSMCs as well as mitochondrial damage. In addition to this, DNA damage can be caused by oxidised lipids and this may also contribute to the dysfunction of endothelial cells, VSMCs, T lymphocytes and macrophages [181].

Oxidative stress has also been associated with the apoptosis or programmed cell death of cardio myocytes [182]. The loss of myocytes via apoptosis has been observed in the infarct regions of myocardium from patients that have suffered from heart failure or a myocardial infarction (MI) [183]. Both in vitro studies and in vivo animal model studies found that apoptosis occurs in response to cardiovascular complications, such as MI, and chronic pressure overload [184]. The common factor in all of these conditions is the generation of oxidative stress and oxidative stress is known to play a role in the initiation of apoptosis [185]. Additionally, the apoptosis of myocytes is inhibited by antioxidants such as vitamin E and SOD, implicating ROS/RNS in the pathological pathways of CVD [186].
Although studies have implicated a role for ROS/RNS in CVD related apoptosis, the exact contribution of oxidative stress in the loss of myocardial function and heart failure remains to be established.

1.9.3. Nitric oxide in CVD

The role of nitric oxide NO’ in vascular homeostasis and signalling has been well characterized. As stated early, NO’ plays a pivotal role in the maintenance of vascular tone and vasoreactivity. In contrast to this distinct role in cell physiology, NO’ can also contribute to CVD pathology. Under certain conditions eNOS (the endothelial isoform of the nitric oxide synthase) becomes uncoupled from a NO’ to an O₂⁻-producing state [187]. The excess O₂ produced can react with NO’ resulting in the formation of excessive ONOO⁻. This reaction reduces the bioavailability of NO’, leading to cardiovascular dysfunction and reduced endothelial vasoregulatory capacity [188]. ONOO⁻ has also been shown to promote the uncoupling of eNOS, propagating the increased ONOO⁻ formation and decreased NO’ bioavailability [189].

1.9.4. Current treatments and antioxidant therapies

The current treatments for comorbid CVD are the same treatments used for CVD independent of COPD, such as β receptor blockers, ACE inhibitors, or angiotensin receptor blockers. However, the benefit of these drugs in COPD patients with CVD is conflicting. Some studies have found that these treatments may be detrimental for COPD patients, worsening the pulmonary symptoms of the disease [190]. However, others have shown that when given to COPD patients with and without comorbid COPD, these treatments have shown notable benefits with regards to airway symptoms [191]. This may be due to the fact that both ACE inhibitors and angiotensin receptor blockers have displayed pleiotropic antioxidant effects in
addition to their antihypertensive effects [192]. Thus, this presents a unique opportunity for dual action therapy using CVD-specific treatments and antioxidants, and understandably warrants further research.

CVD and COPD also overlap with regards to their non-pharmacological management. Cardiovascular modifying factors such as physical activity and diet modification are reported to decrease hospital readmission for COPD [193]. The recognition of the importance of oxidative stress in CVD has led to the fervent use of antioxidants in the treatment and prevention of the disease, however, the results of prospective, randomized clinical trials have been generally disappointing [194, 195]. In contradiction, studies have shown that antioxidant therapy is beneficial in non-comorbid hypertension, atherosclerosis, ischemic heart disease, cardiomyopathies and congestive heart failure [173]. It should be noted that almost all antioxidant clinical studies only explored the effectiveness of traditional scavenging antioxidants such as vitamin E.

With regards to antioxidant treatments, the protective role of exercise in preventing oxidative stress is noteworthy. Acutely, it is known that exercise causes oxidative stress [196]. However, exercise also leads to longer-term activation and enhanced synthesis of antioxidants and antioxidant enzymes (e.g. SOD, Gpx-1), as well as decreasing oxidant production [197]. This may explain the links between prolonged exercise and the beneficial effects to both COPD and CVD patients, and highlights the potential benefits of targeting oxidative stress in comorbid CVD. To our knowledge, the effectiveness of antioxidant therapy on cardiac structure and function has not been explored in murine models of COPD. Additionally, current non-invasive techniques of measuring cardiovascular morphology and function are inadequate for measuring changes in the right ventricle of rodents in a non-terminal manner, limiting in vivo experimental design.
Overall, it appears that targeting oxidative stress with antioxidant enzyme modifying treatments may have great potential in COPD and comorbid CVD by providing beneficial effects with regards to both the pulmonary and cardiovascular aspects.

1.10. Novel antioxidant approaches for the treatment of COPD

Besides never smoking, smoke cessation is the only effective method for preventing the onset and progression of COPD. The progression of airway inflammation, increased oxidative stress, and protease burden even months/years after cessation, and non-responsiveness to glucocorticosteroids have been documented as therapeutic challenges for the treatment of COPD \[33\]. Because of this, there is a lack of effective treatments for COPD and its comorbidities.

As stated throughout this review, tissue injury and inflammation as a result of oxidative stress are common to COPD and many of its comorbidities. A common theme throughout this review is the similarity and interplay between the pathological mechanisms of each condition and it is this similarity that provides a unique therapeutic opportunity (Figure 6). Treatment with antioxidants may be able to progressively prevent and treat multiple diseases by supressing the generation of ROS/RNS, neutralizing oxidants or both. Due to the overwhelming evidence implicating oxidative stress in the pathogenesis of COPD and its comorbidities, it is only logical to consider antioxidant intervention in this patient population. It is important that such interventions not only neutralize the excessive ROS and RNS generated, inhibit peroxidation of lipids, and the subsequent inflammatory response, but to also identify the source of these oxidants and inhibit their generation \[53\]. Due to the imbalance in oxidants and antioxidants, this can be achieved by two approaches: increasing the endogenous antioxidant enzyme activity via enzyme modulators/mimetics, or
by replenishing the depleted non-enzymatic defences through dietary or pharmacological means.

It should be noted that typical radical scavenging treatments, such as Vitamin E and other dietary antioxidants, have shown minimal improvements in either COPD or the comorbidities outlined in this review. This may be due to the dose, route of administration, or the specific antioxidant given and thus more research is required to determine the efficacy of these compounds with certainty. In addition, traditional antioxidants act in a “sacrificial manner,” due to the fact that once they have scavenged a radical, they are essentially consumed.

Undoubtedly, the most damaging of all the ROS/RNS are the hydroxyl radical and peroxynitrite. These species are extremely reactive and indiscriminate to the point that they basically react with the first substrate they come into contact with. Knowing this, the most effective form of protection would be to prevent their generation in the first place and this cannot be performed by traditional antioxidants (scavengers). Therefore, a pharmacological approach that inhibits the production of oxidant species is required. In addition to this, it may also prove beneficial to use combination therapy and treat patients with the pharmacological enzymatic antioxidants (e.g. NOX inhibitors) whilst also supplementing them with dietary antioxidants (e.g. vitamin E supplements).
Figure 6. Points of therapeutic intervention in COPD and its comorbidities.

Oxidative stress indiscriminately damages a number of tissues and, as such, has been implicated in the onset and development of multiple diseases regardless of COPD diagnosis. Using novel antioxidant therapies it may be possible to treat COPD, AECOPD and comorbidities of COPD. Inhibiting the generation of ROS and RNS with pharmacological compounds could reduce lung and systemic inflammation, reduce the severity and progression of COPD and comorbidities, improve the quality of life of patients, and increase their survival.
1.10.1. Novel antioxidants

As shown on the ROS cascade (Figure 2), there are 3 key enzymes that can be targeted in order to reduce oxidative stress; NOX, SOD, and Gpx/Cat. These enzymes can either be inhibited to reduce their activity (e.g. NOX inhibitor - apocynin) or amplified/mimicked to increase their activity (e.g. Gpx mimetics – ebselen). Outlined below are just some of the enzymatic compounds that may be of therapeutic benefit to COPD patients (see Table 1).

### Table 1. Various antioxidant compounds that have been studied in the context of COPD and/or its comorbidities.

Pre-clinical and clinical tests are investigating, or have investigated, the effectiveness of intervention with various small-molecular weight antioxidant compounds that target oxidant generation, or quench inflammation/cigarette smoke-derived oxidants and aldehydes. Refer to Rahman & MacNee (2012) for an in-depth analysis of these various compounds.
1.10.2. NOX inhibitors

As stated earlier, NOX-1 and -2 are the primary generators of $O_2^-$ and are responsible for the initiation of the ROS cascade (Figure 2). NOX is a unique target in the sense that inhibiting the activity of the enzyme would not only reduce the generation of a single oxidant species but also reduce the generation of all other ROS and RNS. Inhibiting NOX would reduce $O_2^-$ production which would result in less available $O_2^-$ for the generation of $H_2O_2$ and $ONOO^-$, subsequently reducing 'OH generation and increasing 'NO bioavailability as a result.

Current studies

There are several NOX inhibitors currently being studied, however the most common is apocynin, which is a NOX inhibitor that preferentially blocks NOX-2 at low doses. It inhibits NOX by preventing the assembly of the NOX enzyme subunits, resulting in the reduced formation of NOX complexes [53, 54]. We have shown that apocynin reduces cigarette smoke-induced lung inflammation in mice (refer to Chapter 4). Additionally, the inhibition of NOX-2 activity ameliorates influenza A virus-induced lung inflammation, indicating that pharmacologically targeting NOX-2 may also have therapeutic potential in seasonal and possibly pandemic influenza infection [199, 200]. Because of this, the possible therapeutic utility of NOX-2 inhibitors may extend to AECOPD. Clinically, COPD patients treated with apocynin had reduced $H_2O_2$ and $NO_2^-$ in their exhaled breath concentrate compared to placebo control [201].

1.10.3. Gpx mimetics

The Gpx family of enzymes, along with catalase, are responsible for the termination of the ROS cascade. There are 8 known isoforms of Gpx (1 to 8) but the most abundant isoform is Gpx-1, which is found in the cytoplasm of almost all mammalian cells and whose preferred
substrate is H$_2$O$_2$ [59]. The main function of Gpx is the reduction of H$_2$O$_2$ to H$_2$O and O$_2$; however, Gpx is also known to reduce lipid peroxides to their corresponding alcohols. Additionally, it has been reported that Gpx-2 is a major cigarette smoke-inducible isoform found in the lung [202]. As with SOD, it is possible to increase the reduction of H$_2$O$_2$ by introducing Gpx (or catalase) mimetics. This would result in the termination of the ROS cascade by increasing conversion of H$_2$O$_2$ to H$_2$O and O$_2$, and subsequently reduced levels of •OH and the decreased levels of lipid peroxides.

**Current studies**

There is currently a limited amount of studies exploring the effectiveness of Gpx mimetics in COPD. When exposed to cigarette smoke, Gpx-1 knockout mice exhibited increased BALF neutrophils, macrophages, proteolytic burden, whole lung IL-17A, and MIP1 mRNA compared with WT mice [203]. When treated prophylactically with the Gpx-1 mimetic ebselen, the cigarette smoke-induced increases in BALF macrophages, neutrophils, proteolytic burden, and macrophage and neutrophil chemotactic factor gene expression were all inhibited in both the WT and knockout mice. In addition, ebselen inhibited established BALF inflammation when administered therapeutically, suggesting that Gpx-1 mimetics may have therapeutic utility in inflammatory lung diseases where cigarette smoke plays a role such as COPD [59]. In addition, we have shown that ebselen caused a reduction in influenza A virus-induced lung inflammation in mice, suggesting that targeting Gpx-1 may be of therapeutic benefit for AECOPD [204].

Ebselen has also been shown to be protective in vivo in disease situations hallmarked by oxidative stress such as diabetes-associated atherosclerosis and cerebral ischaemia–reperfusion injury [205]. Additionally, ebselen has been used in clinical trials of acute
ischaemic stroke and was found to improve the outcome of patients when administered within 24 hours of stroke [206].

1.11. Hypothesis and Aims of this Thesis

COPD is a major incurable global health burden and is the 4th largest cause of death in the world. Exacerbations are a common occurrence in COPD patients and contribute mainly to morbidity, death and health-related quality of life. Comorbid diseases, in particular cardiovascular disease and skeletal muscle wasting, are also common in COPD and potentiate the morbidity of COPD, leading to increased hospitalisations, mortality and healthcare costs. Current treatments have limited efficacy and fail to modify the factors that initiate and drive the long-term progression of COPD, its exacerbations and its co-morbidities. In addition, no pharmacological treatment has been shown to reduce the risk of death in COPD in prospective clinical trials. Moreover, sophisticated non-invasive methods of assessing cardiac function and morphology to explore cardiovascular changes in experimental rodent (and in particular mice) models of COPD are lacking.

It is now evident that increased oxidative stress within the local lung microenvironment is a major driving mechanism in the pathophysiology of COPD and that it may directly influence peripheral organ (e.g. heart and skeletal muscle) behaviour in a ‘COPD-specific manner’. Therefore, I hypothesise that targeting oxidant-dependent mechanisms that drive COPD and its co-morbidities may have great therapeutic potential in treating both pulmonary and extrapulmonary manifestations of the disease. Thus, the primary aims of this thesis were:
Aim 1: To determine if the Gpx-1 mimetic ebselen could improve both lung inflammation and skeletal muscle wasting in an in vivo model of COPD.

To test this aim, we explored the effects of ebselen in an 8-week sub-chronic cigarette smoke exposure protocol in 8-week old Balb/C mice. To assess the impact of ebselen on lung inflammation, we measured inflammation in the BALF and cytokine and chemokine mRNA expression in the lung tissue. To assess the impact of ebselen on skeletal muscle, we measured contractile function of the tibialis anterior, whole skeletal muscle tissue weight, hypertrophy, atrophy, and inflammatory mRNA expression in the tibialis anterior and gastrocnemius, and oxidative damage in the tibialis anterior.

Aim 2: To determine if the NADPH oxidase 2 inhibitor apocynin could improve both lung inflammation and skeletal muscle wasting in an experimental model of COPD.

To test this aim, we explored the effects of apocynin in an 8-week sub-chronic cigarette smoke exposure protocol in 8-week old Balb/C mice. To assess the impact of apocynin on lung inflammation, we measured inflammation in the BALF and cytokine and chemokine mRNA expression in the lung tissue. To assess the impact of apocynin on skeletal muscle, we measured contractile function of the tibialis anterior, whole skeletal muscle tissue weight, hypertrophy, atrophy, and inflammatory mRNA expression in the tibialis anterior and gastrocnemius, and oxidative damage in the tibialis anterior.

Aim 3: To design, develop and validate a non-invasive method of assessing cardiac function and morphology to explore cardiovascular changes of COPD in experimental rodent models.

To test this aim, we adapted existing transthoracic echocardiographic views to examine right ventricular function and morphology in Wistar Kyoto rats. Functional and morphological
parameters were measured and validated by comparing values derived from traditional
echocardiographic techniques and measurements taken during dissection. The viability of
strain analysis using this modified view was also investigated.
Chapter 2: General Methods, Materials and Experimental Design
2. Methods

This general methods chapter describes in detail the various materials and experimental techniques used throughout this thesis. Chapter-specific information can be found in the methods sections of each respective chapter.

2.1. In vivo animal studies

2.1.1. Mice

Eight-week old male Balb/c mice (Animal Resource Centre, Perth, WA) were housed at 20°C in sterile micro-isolator cages under a 12:12h light dark cycle. Mice were given ad libitum access to food and water. Experimental protocols and procedures were approved by the Animal Experimental Ethics Committee of the University of Melbourne (AEC 1413348, 1112185, 1212675), and the RMIT Animal Ethics Committee (AEC 1521).

2.1.2. Rats

300gm Male Wistar Kyoto Rats (Animal Resource Centre, Perth, WA) were house at 20°C under a 12:12h light dark cycle. Rats were given ad libitum access to food and water. All experimental protocols and procedures were approved by the Animal Experimental Ethics Committee of the University of Melbourne (AEC 1212675).

2.2. Experimental procedures

2.2.1. Cigarette smoke exposure

Briefly, mice were placed in an 18 L perspex container in a class II biosafety cabinet, and were exposed to the smoke produced by 3 cigarettes (Winfield Red, ≤16 mg of tar, ≤1.2 mg of nicotine, and ≤15 mg of carbon monoxide; Philip Morris, Melbourne, Australia,), 2 times a day, 5 days per week (10:00AM, 4:00 PM) over a 56 day period as previously described
Sham-exposed mice were handled identically, but exposed to room air instead of cigarette smoke

2.2.2. Chronic cigarette smoke exposure and apocynin groups

Following a 1-week acclimatisation period, mice were divided into four groups (n=8 per group) that were matched for body weight. The groups were allocated as sham + vehicle (SV), sham + apocynin (SA), cigarette smoke + vehicle (CSV) and cigarette smoke + apocynin (CSA). Body weight was measured 3 times each week (Monday, Wednesday, Friday) at 10:00AM during the experimental period. Food intake was measured daily by weighing the food remaining in the animal cage and subtracting from the previous day’s amount. This amount was divided by the number of animals per cage to determine the average intake per mouse per day.

2.2.3. Chronic cigarette smoke exposure and ebselen groups

Following a 1-week acclimatisation period, mice were divided into four groups (n=10 per group) that were matched for body weight. The groups were allocated as sham + vehicle (SV), sham + ebselen (SE), cigarette smoke + vehicle (CSV) and cigarette smoke + ebselen (CSE). Body weight was measured 3 times each week (Monday, Wednesday, Friday) at 10:00AM during the experimental period. Food intake was measured daily.

2.2.4. Apocynin preparation and administration

Mice were treated with apocynin (5 mg/kg; Sigma-Aldrich, Sydney, Australia) or vehicle (0.1% DMSO in Saline) via intraperitoneal (i.p) injection at 9 am per weekday throughout the 56 day period. In order to minimize DMSO content in the drug preparation, apocynin was dissolved in 10 μL of DMSO. This solution was then mixed with 9990 μL of saline resulting in a 0.1% DMSO mixture.
2.2.5. Ebselen preparation and administration

Mice were treated with ebselen (5 mg/kg; Sigma-Aldrich, Sydney, Australia) or vehicle (5% CM-Cellulose in PBS) via oral gavage 5 days per week throughout the 56 day period.

2.3. Sample collection

On day 56 mice were killed by an anaesthetic overdose using pentobarbitone (mg/kg) prepared in sterile saline. Blood was collected from the abdominal vena cava, transferred into a Microvette® collection tube coated with lithium heparin (Sarstedt, Germany), and spun at 10,000 x g rpm for 5 min to separate the plasma. Plasma was stored at -80 °C for later analysis. Bronchoalveolar lavage fluid (BALF) was collected from each mouse by flushing with a 400ml aliquot of PBS, followed by three 300 μL aliquots of PBS, as previously described [208]. Thymus, spleen, kidney, were removed and weighed. The lungs, the left side hind limb skeletal muscles (tibialis anterior (TA), soleus, plantaris, gastrocnemius), testicular white adipose tissue (WAT) and retroperitoneal WAT were removed, weighed, snap-frozen and stored at -80 °C for subsequent analysis. The right side hind limb skeletal muscles were removed, weighed, embedded in OCT and frozen in melting isopentane, and stored at -80 °C for subsequent analysis. The liver was also removed and weighed, with the largest lobe snap-frozen and stored at -80 °C for subsequent analysis.

2.4. Measures of lung inflammation

Viable cells in the BALF were counted using the fluorophores ethidium bromide and acridine orange (AO/EB), on a Nikon Eclipse E600. Cytospins were prepared using 100 μL of BALF at 400 x g rpm for 10 min using a Cytospin 3 (Shandon, UK). Cytospin preparations were stained with DiffQuik (Dade Baxter, Australia), and 500 cells per slide were counted and differentiated into macrophages, neutrophils and lymphocytes using standard morphological
criteria. BALF was centrifuged and the supernatant was stored at -80 °C for subsequent analysis.

2.5. Measures of systemic inflammation and oxidative stress

The protein concentrations of mouse CRP and 8-isoprostan in serum were determined using commercially available ELISA kits. RayBiotech (GA, USA) ELISA kits were used to measure Mouse CRP. OxiSelect™ 8-iso-Prostaglandin F2α ELISA kits were used to measure 8-isoprostan (Cell Biolabs, Inc., CA, USA). Measurements were made according to manufacturer’s specifications.

MDA in mouse serum was measured as marker as of systemic oxidative stress. The concentration of MDA was measured using an OxiSelect™ TBARS Assay Kit (Cell Biolabs, Inc., CA, USA) according to manufacturer’s specifications.

2.6. Western blotting and oxyblot

Western Blot

2.6.1. Tissue homogenization

Tissue was ground via mortar and pestle and 20 mg was resuspended with 500 μL of RIPA lysis buffer (containing 1% B-mercaptoethanol and 1% protease inhibitor). The samples were homogenized with syringe and 21G needle 10-20 times, and incubated on ice for 30 minutes, unexposed to light. The samples were then centrifuged for 10 minutes at 14000 x g rpm, 4°C. The supernatant was collected for immediate use or stored at -80°C.

2.6.2. Protein quantification

Protein concentrations were determined through use of commercially available colorimetric bi-cinchonnic acid (BCA) protein kit (Thermo Fisher Scientific, Melbourne, Australia). Tissue lysate was diluted in lysis buffer in ranges of ½ up to 1/20, then 200 μL BCA reagent
mix (50 parts reagent A to 1 parts reagent B) was added to detect concentration of protein in
the each sample. Solutions were mixed and incubated at 37°C for up to 30 mins or when
nearing saturation (whichever occurred first) prior to the determination of absorbance by
spectrophotometry at 562 nm using a clariostar microplate reader (BMG LabTech, VIC,
Australia). With each BCA assay, varying dilutions of BSA protein were included to create a
standard curve between the ranges of 0 μg/mL to 2.0 μg/mL. Once protein concentrations
were determined, samples were normalised to contain the equal amounts of total protein.

2.6.3. SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

10% Acrylamide gels were made using TGX™ and TGX Stain-Free™ FastCast™
Acrylamide Kits and Starter Kits (Bio-Rad, CA, USA). For a single gel, 3 mL of resolver A
and 3 mL of resolver B solutions were combined as per manufacturer’s specifications. 3μL of
TEMED and 30 μL of freshly made 10% APS was added to the resolver solution and mixed
thoroughly. The solution was dispensed into the cassette and filled to 0.5-1 cm below the
bottom of the teeth on the gel comb. The stacking solution was immediately prepared by
combing 1 mL of stacker A and 1mL of stacker B as per manufacturer’s specifications. 2 μL
of TEMED and 10 μL of freshly made 10% APS was added to the stacker solution and mixed
thoroughly. The solution was dispensed slowly into the cassette (on top of the resolver
solution). The gel comb was aligned and inserted into the cassette carefully in order to
prevent the trapping of air under the comb teeth. The gel was allowed to polymerize for 45
minutes before electrophoresis. 6 μL of each sample supernatant was loaded into a 10%
acrylamide gel and the gels were run at 200V for 60 minutes in running buffer.

2.6.4. Immunoblotting

Following the separation of protein by SDS-PAGE, proteins were then transferred from the
gels to methanol-activated polyvinylidene fluoride (PVDF) transfer membranes (Bio-Rad
Laboratories Inc., USA) at 260mA for 7 minutes. Non-specific binding was blocked with 5%
skim milk in TBS for 1 hour at a room temperature. The membranes were then incubated in the appropriate primary antibodies for 2 hours at the room temperature or overnight at 4°C. Membranes were washed with 1x TBST for 4 times (5 minutes each time) to remove any unbound antibodies, followed by incubation with an appropriate secondary antibody (1:1000 dilution in 1x TBST) for 2 hours at the room temperature. The membranes were again washed with 1x TBST for 4 times (5 mins each time). The bound antibody was detected using a chemiluminescence system with Western Lighting Ultra Solution (Perkin Elmer, NEL113001EA). The membranes were exposed to ChemiDoc (Bio-Rad Laboratories Inc., USA) capturing images for a sufficient time. Quantitative densitometry analysis of interested bands was performed using Image Lab software (Bio-Rad Laboratories Inc., USA). Relative protein expression was normalised by the band intensity of β-actin as an internal control.

2.6.5. Oxyblot

The commercially available OxyBlot Protein Oxidation Detection kit (Merck, Massachusetts, USA) was utilized for immunoblot detection of carbonyl groups introduced onto proteins by oxidative reactions. 5 μL of each supernatant was used, with 2 μL of 4x loading buffer added and then was boiled at 90°C for 5 minutes. 10μL of supernatant was transferred into 1.5 mL tubes, and 10 μL of 12% SDS was added to each sample. 10 μL of each supernatant-SDS mixture was transferred into another 1.5 mL tube to act as a negative control. The sample was derivatised by adding 10 μL of 1x 2,4-Dinitrophenylhydrazine (DNPH) solution (diluted in ddH₂O), while adding 10 μL of 1x derivatisation-control solution to the negative control. The samples were incubated at RT for 15 minutes. 7.5 ul of Neutralization solution was added to each sample. 10 μL of each sample was loaded into a 10% acrylamide gel and the samples were subjected to SDS-PAGE electrophoresis as stated in section 2.6.3. The resolved proteins were transferred onto a PVDF membrane using a Trans-Blot SD transfer cell (Bio-Rad, CA, USA) at 200V for 7 min. After transfer, PVDF membranes were incubated in blocking
solution for 1 h at room temperature. The carbonylated proteins were then bound with primary antibodies and subsequently a secondary antibody as per manufacturer’s instructions. The bound antibody was detected using chemiluminescence as stated in section 2.6.4.

2.7. RNA extraction and RT-qPCR

2.7.1. Sample crushing
Lung samples were crushed to a fine powder in liquid nitrogen with a mortar and pestle, transferred to a fresh 2 mL safe lock tube and stored at -80°C. The crushed tissue was added to either trizol or RNaseasy lysis buffer, depending on the ribonucleic acid (RNA) extraction method used, and subsequently homogenised by passing 10-20 times through a 21G needle with a 1 mL syringe. Gastrocnemius and tibialis anterior samples were crushed as stated above and then homogenised via syringe and needle or disrupted and homogenised using a Tissue Lyser (Qiagen, Australia) according to the manufacturer’s instructions.

2.7.2. RNA Extraction

Trizol RNA extraction
15 mg of crushed tissue was homogenized in 500 μL trizol (Qiagen, Australia) via syringe and needle. Chloroform (200 μL, VWR, #22711324) was added to each homogenate and the mixture was capped securely and vigorously hand inverted for 15 seconds followed by incubation at room temperature for 5 mins. The homogenates were subsequently centrifuged at 12000 x g rpm for 15 mins at 4°C. The mixture was separated into a lower red, phenol chloroform phase containing protein, an interphase containing DNA, and a colourless upper aqueous phase containing RNA. Only the RNA-containing upper aqueous phase was transferred to a set of fresh micro-centrifuge tubes, and mixed with 500 μL of isopropanol (Sigma-Aldrich, Sydney, Australia, #J9516) before another centrifuge at 12000 x g rpm for 10 mins at 4°C. The RNA precipitate formed a gel-like pellet on the bottom side of the tube after centrifugation. The supernatant was removed and the pellet was washed once with 1mL
75% ethanol (Merck, #1.07017.2511). The sample was then mixed by vortexing and centrifuged at 7500 x g for 5 mins at 4°C. The supernatant (ethanol) was removed and the RNA pellet was air dried. At the end of the procedure, the pellet was dissolved in Ambion® DEPC-treated water (Invitrogen, #AM9916, 50 µl for each sample) for RNA concentration determination.

**RNeasy Plus kit RNA extraction**

Samples were crushed as per section 2.7.1 and total RNA was extracted from lung and skeletal muscle samples using an RNeasy Plus kit (Qiagen, Australia), according to the manufacturer’s instructions. Skeletal muscle samples were subsequently incubated in proteinase K (Qiagen, Australia) for 10 min at 55 °C, prior to RNA extraction.

**2.7.3. Measurement of RNA yield**

RNA concentration was quantified by using a NanoDrop Spectrophotometer (Eppendorf Thermo Fisher Scientific, Australia) using an absorbance ratio of 260/280 nm (A260/280). The NanoDrop Spectrophotometer was initialized using 1.0 µL of DEPC water, which was also used as a blank. Each RNA sample (1.0 µL) was loaded onto the sampling platform for the measurement of RNA concentration. Total RNA from samples were reverse transcribed to cDNA (Applied Biosystems High Capacity RNA-to-cDNA kit, USA) according to kit manufacturer’s specifications.

**2.7.4. Quantitative polymerase chain reaction**

qPCR was performed using mouse-specific TaqMan® Gene Expression Assays (Applied Biosystems), using either an ABI 7900HT Sequence Detection System or Quantstudio 7 Flex Real-Time PCR System (Applied Biosystems). Samples were assayed in triplicate. Fold change was determined in comparison to the sham control group, after standardising to GAPDH (housekeeping gene), using the established Delta-Delta C_\text{T} method [209].
2.8. Functional analysis of live animals

2.8.1. Muscle Function

Mouse Grip Strength

Forelimb and total limb grip strength were determined using a grip strength meter (Columbus Instruments, Columbus). Each mouse was held by the base of their tail and suspended above a grip ring. After 3 seconds, the mouse was gently lowered towards the ring and allowed to grasp the ring with its forepaws for forelimb measurement or all paws for total limb measurement. The mouse’s body was lowered to a horizontal position, and the animal’s tail was pulled until the mouse broke its grasp from the ring. The mean force was measured with an electronic pull strain gauge connected directly to the grasping ring. Maximal force obtained from five replicates was used as the dependent measure.

In Situ Muscle Function

Animals were anaesthetised using ketamine/xylazine until pedal pinch reflex was no longer present. The foot of the animal was held in position and secured to a metal plate using elastic bands. A small skin incision was made at the front of the ankle and skin was blunt dissected up to the knee to reveal the tibialis anterior. A gap was made underneath the TA tendon and small incisions were made on each side of the tendon fascia until the tendon was released and freed all the way down below the ankle joint. A 8-10 cm long piece of suture thread was fed under the tendon and a knot was tied on the front of the tendon near the myotendinous junction. Another knot was tied on the underside of the tendon. This was repeated a few mm distal to the first thread with a second piece of 8-10cm long suture thread. The TA tendon was cut as distally as possible and folded over the knotted threads. The threads of the lower knot were placed so they pointed towards the knee. The threads of the upper knot were then used to tie a knot in front of the tendon and threads of the lower knot, encompassing them in a tight bundle. A reverse knot was then tied to secure the tendon and thread bundle. The
threads from the lower knot were then cut off. A scalpel blade was run along the edge of the tibia, releasing the muscle from the fascia and freed off the bone until just below the knee. A hole was made behind the patellar tendon.

The mouse was transferred to the heated platform of the Aurora system (1300A: 3-in-1 Whole Animal System – Mouse, Aurora Scientific, Canada) and secured using the foot clamp. The upper leg was secured by passing a needle behind the patellar tendon and into the heated platform. The remaining threads attached to the TA were used to tie a knot through the hole in the force transducer arm, securing the TA to the force transducer. The two fine electrodes were inserted into the belly of the TA muscle and the muscle was periodically bathed in warm saline through out the testing.

2.8.2. Transthoracic Echocardiographic Analysis of Ventricular Size and Function

Male WKY rats (300-350g) were anesthetized with 2.5% isoflurane, spontaneously ventilated and placed on a heated pad in the semi-left lateral position with upright tilt, suitable for echocardiographic examination. Pulse rate, oxygen saturation and temperature were continuously monitored. After shaving, a sequential examination of the left and right ventricles was performed using a Vivid E9 with i13-L (6-14MHz) linear array transducer (GE Vingmed Ultrasound AS, Horten, Norway). Each study imaged the parasternal long axis (PLAX) and mid-papillary short axis (SAX) views of the left ventricle (LV), and the modified Scherrer-Crosby (SC) and aortic right ventricle outflow tract (RVOT)/pulmonary artery SAX view of the right ventricle (RV) (see below, Figure 29). LV morphology was assessed for interventricular septal (IVS) wall and posterior wall (PW) thickness, LV end-diastolic chamber dimension (LVEDD), LV end-systolic chamber dimension (LVESD), fractional shortening (FS), LV end-diastolic chamber area (LVEDA), LV end-systolic
chamber area (LVESA) and fractional area change (FAC). RV chamber width was assessed at mid-chamber in the SC view (R1) and RVOT width measured in the aortic SAX view (R2). In this view the main pulmonary artery diameter (PAφ) was also measured. RV wall area was measured in both SC and RVOT SAX views calculated from the difference between traced epicardial and endocardial areas [210]. Right ventricular ejection was measured in the aortic SAX view at the proximal MPA (PA diameter) using 2D-Doppler and the waveform analyzed for pulmonary artery velocity-time integral (VTI) and time to peak ejection (PAAT), Pulmonary artery systolic pressure (PASP) was not assessed (TR jet not examined).

2.8.3. Strain Analysis

Real-time imaging loops of ventricular motion during the cardiac cycle were recorded for off-line analysis. The RV was imaged in the adapted SC view and the LV in the mid-ventricular SAX view. High frame rates necessitated manual tracing of epicardial and endocardial walls for 2D-strain analysis (Echopac, version 2011, GE Healthcare, Sydney, Australia). Segmental and global wall motion was then analysed.

2.8.4. Injection of Agarose Gel and Tissue Collection

Animals were euthanized using inspiratory CO₂, the heart immediately excised and injected with KCl. To correlate RV chamber size and wall thickness with echocardiographic measurement agarose gel (2%, ScientifiX, Australia) was injected into the RV chamber and left to fix at room temperature. The gel was suspended in Hank’s Balanced Salt Solution 40 mM HEPES (sHBSS). 10 minutes following injection, the RV wall was excised from the heart, exposing the hardened agarose gel mould of the RV chamber (see below, Figure 30). The RV free wall was then freely dissected away from the septal wall and atrio-ventricular valves. Both the agarose gel mould and RV free wall were weighed.
2.8.5. Parameters measured using TTE:

Left Ventricle (LV) Dimension Parasternal Long Axis (PLAX):

- LV Internal Diastole Diameter (LVIDD)
- LV Internal Systole Diameter (LVISD)
- Posterior Wall Thickness (PWϕ)
- Interventricular Septum Thickness (IVSϕ)

LV Dimension Parasternal Short Axis (PSAX)

- LV End Diastolic Area (LVEDA)
- LV End Systolic Area (LVESA)

Right Ventricle (RV) Aortic Valve (AoV)

- RV Out flow tract (RVOT) Proximal Distance (RVOT prox ϕ)
- RVOT distal distance (RVOT distal ϕ)
- Pulmonary Artery diameter (PAϕ)
- RVOT Epithelial Area (RVOT Epi)
- RVOT Endothelial Area (RVOT Endo)

RV Sheer-Crosby (SC)

- RV Epithelial Area (RV Epi)
- RV Endothelial Area (RV Endo)
- RV Distance (RV4)

Echocardiographic measurements in this study included:

- Fractional Shortening (FS) = LVIDD-LVISD/LVIDD
• Fractional Area Change (FAC) = LVEDA - LVESA/LVEDA
• LV volume/Stroke volume (SV) = based on geometric truncated ellipsoid model[211]
• RV Free Wall Area = Epicardial area – endocardial area
• RVOT Free Wall Area = Epicardial area – endocardial area
• RV ejection stroke volume RVESV = PA area * VTI * HR, where PA area is the cross-sectional area of the main pulmonary artery, VTI is the area beneath the PA pulsed-wave velocity waveform or velocity-time integral and HR is heart rate.

2.9. **In vitro cell culture studies**

2.9.1. **C2C12 mouse skeletal muscle cells**
To explore and assess the mechanisms underlying the effects of cigarette smoke and ROS on skeletal muscle, C2C12 mouse skeletal muscle cells were cultured. This cell line was chosen as it is well established in existing literature for *in vitro* studies of skeletal muscle.

All cell culture experiments were conducted according to the Standard Operating Procedures for Physical Containment Level 2 (PC2) Laboratory area (Module B, Module D, Building 223, RMIT University, Melbourne, Australia) using established facilities including Class II Biohazard Safety cabinets and CO₂ incubators.

2.9.2. **C2C12 storage and handling**
C2C12 murine myoblasts were obtained from ATCC (Manassas, VA, USA) and stored in 2 mL cryovials (Corning, Melbourne, Australia) at -80°C freezer (short-term storage) or in liquid nitrogen (long-term storage). When required, the cryovials were quickly defrosted at 37°C in a water bath with a constant and moderate agitation until fully thawed. Vials were immediately disinfected with 80% (v/v) ethanol and opened in an UV-disinfected cell culture
hood (EuroClone, Pero, Italy) before the thawed cells were slowly pipetted into a sterile 15 mL tube (Falcon, Melbourne, Australia) containing 9 mL pre-warmed growth medium and transferred into a T75 culture flask (Corning, Melbourne, Australia). Cells were observed under the microscope and cell viability was assessed using standard morphological criteria. The flask was then placed in an incubator at 37°C in 5% CO₂. After overnight incubation, the cells were re-examined and assessed for viability.

2.9.3. General cell culture procedures

C2C12 myoblasts were grown in vitro in growth media, which consists of Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 mmol (per 500 mL) glucose, glutamine, sodium pyruvate and supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. The cells were grown in a T75 flask and passaged at 1/10 when cells were nearing or at 100% confluency.

Splitting C2C12 cells

Media was removed from the flask and discarded. Cells were rinsed with 10 mL of sterile PBS, which was then discarded. 2 mL of trypsin + EDTA was added and the flask incubated in a 5% CO₂ incubator at 37°C for 5 minutes or until cells no longer adhered to the flask. 8 mL of DMEM was added to neutralize the trypsin and resulting in a total volume of 10 mL. 1 mL of this mixture was added to another flask containing 15 mL of DMEM, resulting in a 1/10 passage. The remaining 9 mL of the mixture was used for plate preparations or discarded. Flasks containing C2C12 myoblasts were discarded at passage number 20.

Differentiation of myoblasts to myotubes

C2C12 cells undergo a myoblast to myotube conversion as they progress from rapidly diving to a confluent and contact inhibited state. This conversion can be induced at confluency
through the use of low-serum media. The C2C12 cells were seeded into a cell culture plate in growth media and allowed to reach 90-100% confluency. The post-confluent cells were washed with PBS and the media was changed to differentiation media which consists of Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 mmol (per 500 mL) glucose, glutamine, sodium pyruvate supplemented with 2% horse serum and 1% penicillin and streptomycin.

**Freezing down cells for long-term storage**

A T75 flask containing adherent cells was first washed in 1x PBS to remove all content of growth medium. This was subsequently removed and 2 mL of 1x trypsin-EDTA solution (ThermoFisher, Australia) added for 1 min. A firm tap to the side of the flask successively detached the cells and the enzymatic action of trypsin-EDTA was stopped with the further addition of 8 mL of growth medium. This cell suspension was centrifuged at 100 x g rpm for 5 mins. After removal of the supernatant, the remaining cell pellet was re-suspended in 0.5 mL of DMEM / 20% FBS / 10% DMSO / 1% Penicillin and Streptomycin to re-suspend cells, and then 1 mL aliquots were prepared in each cryovial. Cells were counted using a haemocytometer to determine the number added per vial. Cryovials were then transferred to a Nalgene® cryo 1°C freezing container (Thermo Fisher Scientific, Australia) filled with 100% isopropanol (Sigma-Aldrich, Sydney, Australia, #I9516), and stored overnight at -80°C. The following day, the vials were placed in liquid nitrogen for long-term storage.

**Counting of cell number**

Cells were counted using a haemocytometer, adding 10 μL of cells suspension between a glass coverslip and the surface of a haemocytometer marked as depicted below. Cells were viewed using microscope (10x or 20x magnification) and the cell numbers in each area were
then calculated (Area 1~4). To find the mean number of cells per Area (4 x 16 corner squares), the sum of Areas 1-4 was divided by 4, and this value was then multiplied by 10⁴ (µL to mL) to provide an estimate for the number of cells per mL.

2.9.4. Cigarette smoke extract preparation

The gas phase extract of cigarette smoke (CSE) was prepared by bubbling 1 cigarette (Winfield Red, ≤16 mg of tar, ≤1.2 mg of nicotine, and ≤15 mg of carbon monoxide; Philip Morris, Melbourne, Australia,) through the required media for desired cell culture assays. The cigarettes were manually pumped (as shown in the schematic below) at a rate of 5 mL/second. Only a single cigarette was combusted per 25 mL of media. The extract was then sterile filtered for use in cell culture.

2.9.5. Treatment of C2C12 Cells

**Hydrogen peroxide**

C2C12 cells were seeded on a 96 well plate and when differentiated, were treated with hydrogen peroxide (30%; Chem-Supply, SA, AUS) at 10, 30, 100, 300, 1000 and 3000 µM
and incubated for 1, 3, 6 and 24 hours before viability was measured using both MTS and MTT assays.

**Cigarette smoke extract**

C2C12 cells were seeded on a 96 well plate and when differentiated, were treated with CSE at 2.5, 5, 10 and 20% CSE solutions prepared as stated previously. The cells were incubated for 24, 48 and 72 hours before viability was measured using an MTS assay.

### 2.9.6. Cell Viability Assays

To assess the viability of cells following exposure to CSE and H$_2$O$_2$, multiple cell viability and cytotoxicity assays were performed. The MTT assay was initially performed but the process was found to be too time consuming at the longer time points stated previously. The MTS assay was used for the majority of the cell viability assays as it was more efficient, easier to perform, and provided results of the same accuracy as the MTT assay.

**Culturing cells for assays**

C2C12 cells were seeded at 10,000 cells per well on a 96-plate, allowed to reach confluency. As per section 2.9.3, the growth media was removed and differentiation media was added, in order to differentiate the C2C12 myoblasts to myotubes. The myotubes were then treated with compounds of interest and incubated as stated previously. Once treatment was complete, existing media was removed from each well, cells were washed with PBS and 100 μL of differentiation media was added to each well.

### 2.9.7. MTT Assay

Vybrant® MTT Cell Proliferation Assay Kit (Thermofisher Scientific, Melbourne, Australia) provides a simple method for determination of cell number using standard microplate absorbance readers. The MTT assay involves the conversion of the water soluble MTT (3-
(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan. The formazan is then solubilized, and the concentration determined by optical density.

**Preparation of solutions**

As per manufacturer’s specifications, a 12 mM MTT stock solution was prepared by adding 1 mL of sterile PBS to one 5 mg vial of MTT (Component A). This was then mixed by vortexing until dissolved. Any undissolved particulate material was removed by filtration if required. Once prepared, the MTT solution could be stored for four weeks at 4°C protected from light. Each 5 mg vial of MTT provided sufficient reagent for 100 tests, using 10 μL of the stock solution per well. 10 mL of 0.01 M HCl was added to one tube containing 1 g of SDS (Component B). The solution was mixed gently by inversion until the SDS dissolved. Once prepared, the solution was used promptly. Each tube makes sufficient solution for 100 tests, using 100 μL per well.

**Labeling of cells using normal MTT protocol**

After fresh differentiated media was added to each well (as stated in 2.9.5) 10 μL of the 12 mM MTT stock solution was added to each well. A negative control of 10 μL of the MTT stock solution added to a well only containing 100 μL of differentiation media. The plate was incubated at 37°C for 2-4 hours depending on the density of the wells. At densities of >100,000 per well, incubation time was reduced to 2 hours. 100 μL of the SDS-HCl solution was added to each well and mixed thoroughly using the pipette. The Microplate was incubated at 37°C for another 4 hours in a humidified chamber. Longer incubations decreased the sensitivity of the assay. Each sample was mixed again using a pipette and absorbance measured at 570 nm.
Labeling of cells using quick MTT protocol

Due to the lengthy incubation times in the standard MTT protocol, a quick protocol was performed according to the manufacturer’s specifications. After labeling the cells with MTT (as described previously) all but 25 µL of medium was removed from the wells. 50 µL of DMSO was added to each well and mixed thoroughly using a pipette. The microplate was incubated at 37°C for 10 minutes. Each sample was mixed again and absorbance was measured at 540nm (instead of 570nm as stated previously).

2.9.8. MTS Assay

The commercially available CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Sydney, Australia) is a tetrazolium-based colorimetric assay for the determination of the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays.

C2C12 cells were seeded at 10,000 cells per well on a 96-plate, allowed to reach confluency and differentiated. The myotubes were then treated with compounds of interest and incubated as stated previously. Once treatment was complete, existing media was removed from each well, cells were washed with PBS, and 100 µL of differentiation media was added to each well. As per manufacturer’s protocol, 20 µL of the MTS assay solution was added to each well and the plate was then incubated at 37°C in 5% CO₂ until desired saturation level was reached, usually ranging between 30 minutes to 2 hours. Absorbance was then measured at 490 nm using a Clariostar 96-well plate reader (BMG Labtech, VIC, Australia).

2.9.9. Extracellular flux assays

To assess the effects of CSE and H₂O₂ on cell respiration, extracellular flux assays were performed using a Seahorse XF²4 Analyzer (Agilent Technologies, CA, USA). The analyzer can be used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells in a 24-well plate format. Respectively, these rates are key
indicators of mitochondrial respiration and glycolysis, providing a complete metabolic profile of cellular metabolic function in cultured cells, islets, and ex vivo samples.

**Culturing of Cells for XF Assay**

Cells were seeded at 10,000 cells per well onto a XF®24 microplate (Agilent Technologies, CA, USA), leaving 4 empty wells (A1, B4, C3 and D6) as per manufacturer’s specifications. The cells were allowed to reach confluency and the growth media was removed and differentiation media was added. When differentiated into myotubes, the cells were treated with CSE and H₂O₂ as described previously.

**Analyzer Preparation on the Day Prior to Assay**

Per manufacturer’s specifications, the analyzer was turned on and warmed overnight (minimum of 5 hours). The sensor cartridge was hydrated in XF Calibrant at 37°C in a non-CO₂ incubator overnight. The experiment template was designed on the XF software on the computer connected to the Seahorse XF®24 Analyzer.

**Preparation of XF Media**

The XF Media was prepared on the day of the assay. 100 mL of XF Base Medium was warmed to 37°C. 0.45 g of glucose (25mM), 1.0 mL of sodium pyruvate (1.0 mM) and 1.0mL of L-glutamine (4 mM) was added to the XF Base Medium. The pH was adjusted to 7.4 using NaOH in small volumes. The medium was sterilized using a 0.2 μm filter and kept at 37°C until required for use.
2.9.10. Treatment of Cells for XF Assay

H$_2$O$_2$

Based on the results acquired from the cell viability assays, cells were treated with 0, 10, 30, 100 and 300 μM concentrations of H$_2$O$_2$ for 1 hour prior to assay (4 wells per concentration). The medium in the wells was removed and replaced with 525 μL of the warmed XF Assay medium prepared previously. The microplate was then placed into a non-CO2 incubator for an hour prior to the Mito Stress Test Assay.

CSE

Based on the results acquired from the cell viability assays, cells were treated with 0, 2.5, 5, 10 and 20% concentrations of CSE for 1 hour prior to assay (4 wells per concentration). The medium in the wells was removed and replaced with 525 μL of the warmed XF Assay medium prepared previously. The microplate was then placed into a non-CO$_2$ incubator for an hour prior to the Mito Stress Test Assay.

2.9.11. Seahorse Mito Stress Test

The Seahorse XF Cell Mito Stress Test (Agilent, CA, USA) provides a complete mitochondrial respiratory profile. The test allows for the determination of: basal respiration, ATP production, proton leak, maximal respiration, and reserve capacity of the cell. The standard protocol described below, was followed as per the manufacturer’s instructions.

Preparation of Assay Compounds

Compounds were prepared on the day of the assay and discarded after assay. The foil pouch was removed from the XF Cell Mito Stress Test Kit box. Each pouch contained enough reagents (Oligomycin, FCCP, and Rotenone/Antimycin A) for one complete microplate. The
compounds were allowed to warm up to room temperature in the sealed pouch for 15 minutes. The contents of each tube were resuspended with the prepared XF Assay Medium as follows:

- Oligomycin (100 μM): 630 μL Assay Medium
- FCCP (100 μM): 720 μL Assay Medium
- Rotenone/AA (50 μM): 540 μL Assay Medium

The 3mL solutions of the resuspended compounds were prepared and loaded into the XF24 ports as follows:

*(Oligomycin):* Final well concentration of 1.0 μM

- 300 μL stock solution in 2700 μL Assay Media
- Loaded 56 μL into port A

*(FCCP):* Final well concentration of 1.0 μM

- 300 μL stock solution in 2700 μL Assay Media
- Loaded 62 μL into port B

*(Rotenone/AA):* Final well concentration of 0.5 μM

- 300 μL stock solution in 2700 μL Assay Media
- Loaded 75 μL into port C

**Running the XF Cell Mito Stress Test**

After the Microplate was incubated for 1 hour and compounds loaded into ports, the hydrated sensor cartridge was calibrated in the XF24 Analyzer. When calibrated, the hydration plate was removed and the Microplate inserted into the machine. The test was then run according to the experimental template designed according to manufacturer’s specifications.
2.10. Statistics

As data were normally distributed, they are presented as grouped data expressed as means ± SE; \( n \) represents the number of mice. Student’s T test was used to assess significance where only two data sets were present. Multivariate analysis was performed via one- or two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons, where appropriate. All statistical analyses were performed using GraphPad Prism for Windows and Mac, versions 6.0-8.1 (GraphPad Software, CA, USA). In all cases, probability levels less than 0.05 (\( P < 0.05 \)) were taken to indicate statistical significance.
Chapter 3: The effect of ebselen in an 8-week cigarette smoke exposure *in vivo* model of COPD
3. Introduction

In this chapter, the effect that treatment with ebselen had on lung inflammation and skeletal muscle wasting in an 8-week smoke exposure model of COPD was explored and all the experimental results are presented and examined in detail. Due to insufficient animal samples, the smoke exposure portion of the study was replicated, however this was completed at another animal facility. We determined that the data sets could not be combined due to variability between the animal facilities (refer to Chapter 6). As both data sets are included in the results section, the first study will be referred to as Study A, and the second referred to as Study B for the purpose of this chapter.

COPD is characterized by persistent airflow limitation and lung inflammation resulting in a progressive decline in lung function [12]. In addition to the pulmonary manifestation of COPD, many systemic manifestations occur in the form of comorbid diseases, such as skeletal muscle wasting [13-15]. Skeletal muscle wasting occurs in approximately 20 to 40% of all COPD patients [17, 111, 112] and is multifactorial in nature. Such factors include inflammation and oxidative stress and these factors can initiate or enhance atrophy in peripheral muscles of patients with COPD [112]. Chronic oxidative stress not only contributes to muscle wasting, but it is also associated with dysfunction in both respiratory and peripheral skeletal muscle.

There are currently no effective drug treatments for skeletal muscle wasting in COPD. Pharmacological treatments that have been explored include, anabolic steroids, growth hormone, other anabolic compounds, bioactive nutrients (e.g. ghrelin, creatine) and antioxidants [212-214]. Most of these treatments have shown little to modest benefits when treating skeletal muscle wasting and weakness in COPD [215-217]. Therefore, new treatments are needed, as limiting muscle wasting in COPD will lead to improved quality of life and
increased survivability of patients. Because of its role in the pathogenesis of comorbid skeletal muscle wasting, targeting oxidative stress may be able to slow the progression or onset of the disease, giving patients an improved quality of life while managing their other symptoms.

As described in chapter 1, glutathione peroxidases (GPxs) are a family of enzymes that catalyze the reduction of $\text{H}_2\text{O}_2$, as well as several other hydroperoxides (such as DNA peroxides and lipid peroxides), into relatively harmless water and alcohols [204, 218]. The ability of Gpx enzymes to eliminate $\text{H}_2\text{O}_2$ is well established and studies have shown that Gpx-1 plays a protective role against oxidative stress [204, 219]. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) is a non-toxic seleno-organic drug that has been comprehensively researched for over three decades [220]. It is now under active investigation for the treatment of various conditions ranging from diabetes to hearing loss [221, 222]. A substantial part of the pharmacological profile of ebselen appears to be due to its action as an antioxidant with a unique mode of action [220]. Traditional thiol antioxidants such as N-acetyl-l-cysteine, act as scavengers of free radicals and thus prevent lipid peroxidation and prevent the secondary reactions in the ROS and RNS cascades triggered by them [198, 223]. In contrast, ebselen is a poor radical scavenger, but is an effective scavenger of organic hydroperoxides, particularly lipid hydroperoxides [224]. Ebselen has also been shown to inhibit a number of enzymes involved in inflammation such as lipoxygenases, nitric-oxide synthases, NADPH oxidase, protein kinase C, and $\text{H}^+$/K$^+$-ATPase [225-228]. Additionally, ebselen has been shown to suppress inflammation in a variety of experimental animal models making the compound a key candidate for the treatment of chronic inflammatory and oxidative conditions such as COPD and its comorbidities [229, 230].
The aim of this study was to investigate whether the Gpx mimetic ebselen protects against cigarette smoke-induced lung inflammation and skeletal muscle wasting in an established subchronic preclinical model of COPD.

3.1. Materials and Methods

3.1.1. Animals

Eight-week old male Balb/c mice were obtained from the Animal Resource Centre Pty. Ltd. (Perth, WA) and housed at 20°C in sterile micro-isolator cages under a 12:12h light dark cycle. Mice were given *ad libitum* access to food and water. Experimental protocols and procedures were approved by the Animal Ethics Committee of the University of Melbourne (AEC 1413348), and the RMIT Animal Ethics Committee (AEC 1521). Given that my supervisor Prof Ross Vlahos moved to RMIT University during my PhD candidature, Study A was performed at The University of Melbourne whereas Study B was performed at RMIT University.

3.1.2. Cigarette Smoke Exposure

Mice were exposed to the smoke produced by 3 cigarettes (Winfield Red; Philip Morris, Melbourne, Australia.), 2 times a day, 5 days per week over a 56-day period as described in section 2.2.3.

3.1.3. Ebselen Administration

Mice were treated with ebselen (10 mg/kg; Sigma-Aldrich, Sydney, Australia) or vehicle (5% CM-Cellulose in PBS) via oral gavage 1 hour prior to their first cigarette smoke exposure of the day. Further details on the experimental design can be found in section 2.1.2.3.
3.1.4. Body Weight and Food Consumption

Body weight was measured 3 times each week (Monday, Wednesday, Friday) at approximately the same time each weighing day. Food consumption was calculated by measuring the amount of food remaining in cages every 24 hours and dividing that by the number of mice in the cages.

3.1.5. Muscle Function

The force-frequency relationship of the tibialis anterior muscle was measured in situ using the 1300A: 3-in-1 Whole Animal System – Mouse (Aurora Scientific, Canada) and further details can be found in section 2.8.1.2.

3.1.6. Blood, BALF and Tissue Collection

On day 56 mice were killed by an overdose of anaesthetic (sodium pentobarbitone, 200 mg/kg prepared in sterile saline), blood collected from the abdominal vena cava, transferred into a Microvette® collection tube coated with lithium heparin (Sarstedt, Germany), and centrifuged at 10,000 x g rpm for 5 min to separate the plasma. Plasma was stored at -80 °C for later analysis. Bronchoalveolar lavage fluid (BALF) was collected from each mouse by flushing lungs with a 400 μL aliquot of PBS, followed by three 300 μL aliquots of PBS. The thymus, spleen, and kidneys were removed and weighed. The lungs, the left side hind limb skeletal muscles (tibialis anterior, soleus, plantaris, gastrocnemius), testicular white adipose tissue (WAT) and retroperitoneal WAT were also removed, weighed, snap-frozen in liquid nitrogen and stored at -80 °C for subsequent analysis. The right side hind limb skeletal muscles (tibialis anterior, soleus, plantaris, gastrocnemius) were removed, weighed, embedded in OCT and frozen in melting isopentane, and stored at -80 °C for subsequent analysis. The liver was also removed and weighed, with the largest lobe snap-frozen and stored at -80 °C for subsequent
analysis. The total number of viable cells, macrophages, neutrophils and lymphocytes in the BALF was determined as described in section 2.2.4.

3.1.7. Quantitative RT-qPCR

In the lungs, the mRNA expression of the following inflammatory cytokines, proteins and enzymes was measured: MMP-9, MMP-12, GM-CSF, G-CSF, TNF-α, CXCL9, IL-6, IL-23, IL-17, IL-1β, MCP-1, and MIP-2α. In the tibialis anterior and gastrocnemius muscles, the mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 were measured. GAPDH was used as the reference gene against all genes measured and all measurements were made using commercially available, validated Taqman primer/probes. In order to measure as many genes as possible while rationing collected samples, preliminary analysis used pooled samples from each animal in each group. As the samples were pooled, only trends could be observed and statistical significance could not be determined. Refer to section 2.7 for RNA extraction and RT-qPCR protocol information.

3.1.8. ELISAs and TBARS

The protein concentrations of IL-6, IGF-1, CRP and 8-isoprostane were determined in serum using commercially available ELISA kits and measured according to manufacturer’s instructions. Further details can be found in section 2.5.

The concentration of MDA was measured using an OxiSelect™ TBARS Assay Kit (Cell Biolabs, Inc., CA, USA) according to manufacturer’s instructions.
3.1.9. Oxyblot

Protein carbonylation, a marker of oxidative stress, was measured in muscle tissue using an Oxyblot Protein Oxidation Detection kit (Merck, Massachusetts, USA). β-actin was used as the reference protein. For further details, refer to sections 2.6.1 and 2.6.2 respectively.

3.1.10. Statistical Analyses

As data were normally distributed, they are presented as grouped data expressed as mean ± SEM; n represents the number of mice. Student’s t test was used to assess significance where only two data sets were present. Multivariate analysis was performed via one- or two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons, where appropriate. All statistical analyses were performed using GraphPad Prism for Windows and Mac (version 6.0-8.1). In all cases, probability levels less than 0.05 (P < 0.05) were taken to indicate statistical significance.

3.2. Ebselen Study A

3.2.1. Assessment of BALF inflammation

As a measure of local inflammation in the lungs, BALF was collected and total cells were counted. A differential cell count was then performed to determine the number of macrophages and neutrophils. Cigarette smoke exposure (6 cigarettes/day, 5 days/week for 8 weeks) significantly increased the total, macrophage and neutrophil number in BALF. However, smoke-exposed mice treated with ebselen had a significant reduction in total cells and neutrophils compared with vehicle treated smoke-exposed mice. Following the 56-day exposure period, the number of total cells in the BALF was 2.36x10^5 ± 2.45x10^4, 1.93x10^5 ± 1.62x10^4, 8.24x10^5±5.05x10^4, and 6.55x10^5±4.93x10^4 cells/mL for the sham + vehicle, sham
+ ebselen, smoke + vehicle, and smoke + ebselen respectively (Figure 7A). The number of macrophage cells was $2.33 \times 10^5 \pm 2.41 \times 10^4$, $1.93 \times 10^5 \pm 1.61 \times 10^4$, $5.79 \times 10^5 \pm 6.20 \times 10^4$, and $5.26 \times 10^5 \pm 3.94 \times 10^4$ cells/mL (Figure 7B), whilst the number of neutrophils was $2.82 \times 10^3 \pm 88.4$, $363 \pm 159$, $2.43 \times 10^5 \pm 4.84 \times 10^4$, and $1.29 \times 10^5 \pm 1.42 \times 10^4$ cells/mL for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen respectively (Figure 7C).

![Graphs showing total cells, macrophages, and neutrophils in BALF.](image)

Figure 7. The effect of ebselen (10mg kg⁻¹) administration on BALF cellularity in mice exposed to air (sham) or after cigarette smoke (smoke) exposure.

BALF cellularity is shown as (A) the total number of cells, (B) macrophages, (C) neutrophils. Data are expressed as mean ± SEM for n=10 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.
3.2.2. **Assessment of cytokine and chemokine mRNA expression in lungs**

Lung tissue was pooled, and RNA extracted. The expression of matrix metalloprotease 9 (MMP-9), MMP-12, granulocyte macrophage colony stimulating factor (GM-CSF), tumour growth factor beta (TGF-β), tumour necrosis factor alpha (TNF-α), C-X-C motif chemokine 10 (CXCL10), interleukin-6 (IL-6), IL-23, IL-17, IL-1β, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 2-alpha (MIP-2α) was measured via qRT-PCR (Figure 8).

Cigarette smoke exposure elevated GM-CSF, MIP-2α, IL-1β, MCP-1, TNF-α, TGF-β, MMP12, IL-23, IL-17A and IL-6 mRNA expression. Additionally, smoke exposure reduced MMP9 expression. Treatment with ebselen reduced in smoke-induced GM-CSF, MIP-2α, MCP-1, TNF-α, TGF-β, MMP12, IL-23, IL-17A and IL-6 expression (all the above excluding IL-1β). mRNA expression of CXCL10 was not affected by cigarette smoke exposure. Due to the pooling of samples, statistical analysis could not be performed for this data.
Figure 8. The effect ebselen (10mg kg⁻¹) administration on pooled lung tissue mRNA expression of inflammatory cytokines and chemokines in mice exposed to air (sham) or cigarette smoke (smoke) exposure

Data are expressed as pooled value from n=10 mice per treatment group. As the samples were pooled, this data could not be statistically analysed.
3.2.3. Impact of smoke exposure on total body weight and food consumption

Mouse body weight was measured each day and food consumption was measured Monday, Wednesday and Friday, with both measurements conducted at approximately the same time per day. Smoke-exposed mice, regardless of treatment, experienced reduced growth compared to their sham counterparts. Surprisingly, smoke-exposed mice treated with ebselen experienced a reduced rate of growth over the vehicle treated group, however this difference was minimal by day 56 (Figure 9A). This is reflected in the final percentage change shown in Figure 9B, which shows that smoke exposure caused a significant reduction in body weight and that the smoke + ebselen group had no improvement in total body weight over the smoke + vehicle group on day 56.

At the end of the 56 exposure period, the average body weights of the mice increased by 16.2±1.1%, 17.7±1.0%, 0.380±1.1%, and -1.14±1.2% in the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen groups respectively to 24.34±0.50g, 25.2±0.63g, 21.2±0.50g *, and 20.7±0.39g**. (Table 2 and Figure 9A).

Food intake was measured to determine if ebselen had an impact on nicotine-induced reductions in appetite [231]. As expected, both smoke-exposed groups experienced a significant reduction in food intake compared to their sham counterparts (Figure 9C). In addition, ebselen did not impact daily food intake. The average daily food intake of the animals was 3.54±0.06g, 3.57±0.05g, 3.12±0.02g, and 3.14±0.02g for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen groups respectively.
Figure 9. The effect of ebselen (10mg kg\(^{-1}\)) administration on total body weight and food consumption in mice after smoke exposure.

(A) Body weight was recorded daily for each group over the 56-day period. (B) Final percentage weight change on day 56. (C) Food intake was calculated by measuring the amount of food remaining in the cages and dividing the difference between days by the number of mice in the cage. Data are expressed as mean ± SEM for n=10 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

3.2.4. Impact of cigarette smoke on organ, skeletal muscle and adipose tissue mass

Lung and heart wet weight

In general, cigarette smoke exposure caused a significant increase in lung weight, which was not reduced by treatment with ebselen. Specifically, compared to the sham + vehicle control, the smoke + vehicle mice lung weight increased by 10.7% and the smoke + ebselen group by
13.7% (Table 2). Cigarette smoke exposure significantly reduced heart weight, which was not improved by treatment with ebselen. Specifically, compared to the sham + vehicle control, the smoke + vehicle mice heart weight decreased by 16.2% and the smoke + ebselen group by 10.9%. This was in contrast to the expected effect of smoke exposure causing an increase in cardiac mass. This is concordant with the decrease in heart mass observed in the apocynin study (refer to Chapter 4). Due to the poor outcomes of the cardiac pilot analysis outlined in Chapter 4, I did not assess cardiac changes in this study and sought to instead develop a model of cardiac dysfunction using cigarette smoke exposure (refer to Chapter 5).

**Skeletal muscle and WAT wet weight**

In general, cigarette smoke exposure significantly decreased the weights of the tibialis anterior, gastrocnemius, and both WAT (Table 2). There was no significant weight loss observed in the plantaris and soleus muscles, however the reduction in soleus weight approached statistical significance (P<0.06). Smoke exposure significantly reduced the weights of the tibialis anterior and gastrocnemius by 9.6% and 9.7% respectively in the smoke + vehicle mice, and 10.14% and 8.9% in the smoke + ebselen mice. Additionally, smoke-exposure significantly reduced the weight of the testicular WAT and retroperitoneal WAT by 36.7% and 47.5% respectively in the smoke + vehicle mice and 41.2% and 51.1% in the smoke + ebselen mice. Treatment with ebselen did not protect mice from this smoke-induced reduction in skeletal muscle and WAT weight.
Table 2. The effect of ebselen (10 mg kg\(^{-1}\)) administration on lung, heart, skeletal muscle weights, and WAT weights in mice after cigarette smoke exposure.

Data are expressed as mean ± SEM for n=10 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sham Vehicle</th>
<th>Sham Ebselen</th>
<th>Smoke Vehicle</th>
<th>Smoke Ebselen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>24.3 ± 0.50</td>
<td>25.2 ± 0.63*</td>
<td>21.2 ± 0.50*</td>
<td>20.7 ± 0.39*</td>
</tr>
<tr>
<td><strong>Tibialis anterior (mg)</strong></td>
<td>55.9 ± 2.3</td>
<td>54.9 ± 1.5</td>
<td>50.5 ± 0.9*</td>
<td>50.2 ± 1.1*</td>
</tr>
<tr>
<td><strong>Soleus (mg)</strong></td>
<td>8.40 ± 0.3</td>
<td>8.80 ± 0.4</td>
<td>7.90 ± 0.3</td>
<td>9.10 ± 0.1</td>
</tr>
<tr>
<td><strong>Gastrocnemius (mg)</strong></td>
<td>136 ± 2.5</td>
<td>130 ± 4.5</td>
<td>123 ± 2.2*</td>
<td>124 ± 2.6*</td>
</tr>
<tr>
<td><strong>Plantaris (mg)</strong></td>
<td>17.3 ± 0.6</td>
<td>17.6 ± 0.4</td>
<td>15.8 ± 0.6</td>
<td>16.7 ± 1.6</td>
</tr>
<tr>
<td><strong>Testicular WAT (mg)</strong></td>
<td>724 ± 56</td>
<td>833 ± 60.7</td>
<td>459 ± 39*</td>
<td>426 ± 37*</td>
</tr>
<tr>
<td><strong>Retroperitoneal WAT (mg)</strong></td>
<td>129 ± 12.1</td>
<td>150 ± 10.5</td>
<td>67.8 ± 7.3*</td>
<td>63.2 ± 5.1*</td>
</tr>
<tr>
<td><strong>Lung (mg)</strong></td>
<td>253 ± 7.0</td>
<td>240 ± 4.2</td>
<td>281 ± 9.6*</td>
<td>288 ± 6.1*</td>
</tr>
<tr>
<td><strong>Heart (mg)</strong></td>
<td>130 ± 3.1</td>
<td>126 ± 3.0</td>
<td>109 ± 9.0*</td>
<td>116 ± 2.8*</td>
</tr>
</tbody>
</table>

3.2.5. Assessment of hind limb and total limb muscle function

On day 53, mouse grip strength collected as a measurement of skeletal muscle dysfunction following smoke exposure. In general, smoke exposure caused a reduction in grip strength in both the forelimb and total limb data sets. Additionally, treatment with ebselen did not improve either of the outcomes. Specifically, forelimb grip strength was 0.186 ± 0.006 N, 0.188 ± 0.006 N, 0.153 ± 0.003 N, and 0.160 ±0.007 N (Figure 10A) and total limb grip strength was 0.266 ± 0.011 N, 0.274 ± 0.012 N, 0.246±0.006 N, and 0.249 ± 0.007 N for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen respectively (Figure 10B). When normalised to body weight, the reduction in grip strength was not significant (Figure 10C and 10D), indicating that the observed loss in grip strength is proportional to the total body weight loss in the animals. In hindsight, a more accurate measurement of normalisation could have been the use of total body length (nose to tail) or tibia length.
Additionally, the changes observed in the raw data may also be a reflection of operator bias as the technique is highly dependent on the researcher. Therefore, to collect specific muscle measurements in the studies that followed, the use of the grip strength meter was discontinued, and muscle function instead measured in situ using a force transducer.

Figure 10. The effect of ebselen (10mg kg\(^{-1}\)) administration on forelimb and total limb grip strength in mice after cigarette smoke exposure.

(A) Forelimb (unadjusted), (B) total limb (unadjusted), (C) forelimb normalised to body weight, and (D) total limb normalised to body weight. Data are expressed as mean ± SEM for n=10 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle
3.3. Ebselen Study B

3.3.1. Assessment of BALF inflammation

In the second study, cigarette smoke exposure (6 cigarettes/day, 5 days/week for 8 weeks) significantly increased the total, macrophage and neutrophil number in BALF (Figure 11). However, smoke exposed mice treated with ebselen had a significant reduction in neutrophils of 51.7% when compared to the smoke + vehicle mice (Figure 11C). As previously observed, ebselen did not significantly reduce the number of macrophages in the BALF (Figure 11B). Following the 56-day exposure period, the number of total cells in the BALF was $1.57 \times 10^5 \pm 1.34 \times 10^4$, $1.18 \times 10^5 \pm 1.24 \times 10^4$, $6.98 \times 10^5 \pm 4.71 \times 10^4$, and $6.42 \times 10^5 \pm 3.82 \times 10^4$ cells/mL for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen respectively (Figure 11A). The number of macrophage cells was $1.57 \times 10^5 \pm 1.52 \times 10^4$, $1.16 \times 10^5 \pm 1.21 \times 10^4$, $4.52 \times 10^5 \pm 3.16 \times 10^4$, and $5.07 \times 10^5 \pm 2.67 \times 10^4$ cells/mL (Figure 11B), whilst the number of neutrophils was $4.29 \times 10^5 \pm 1.07 \times 10^3$, $168 \pm 2.14 \times 10^3$, $2.76 \times 10^5 \pm 1.03 \times 10^4$, and $1.33 \times 10^5 \pm 1.92 \times 10^4$ cells/mL for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen respectively (Figure 11C).
**Figure 11. The effect of ebselen (10mg kg⁻¹) administration on BALF cellularity in mice after smoke exposure.**

BALF cellularity is shown as (A) the total number of cells, (B) macrophages, (C) neutrophils. Data are expressed as mean ± SEM for n=12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.

### 3.3.2. Impact of smoke exposure on total body weight and food intake

As with Study A, mouse body weight was measured each day and food consumption was measured Monday, Wednesday and Friday, with both measurements conducted at approximately the same time per day. Smoke-exposed mice, regardless of treatment, experienced reduced growth compared to their sham counterparts. As seen in Figure 12A, smoke-exposed mice treated with ebselen experienced a slightly improved rate of growth over the 56 day period compared to the vehicle treated group. However, when examining the final percentage change shown in Figure 12B, there was no significant improvement in total body weight as a result of treatment with ebselen. At the end of the 56 day exposure period,
the average body weights of the mice increased by 11.4 ± 1.0%, 13.6 ± 0.9%, 2.99 ± 1.3%,
and 5.21 ± 1.1% in the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke +
ebselen groups respectively to 24.3 ± 0.50g, 25.2 ± 0.63g, 21.2 ± 0.50g *, and 20.7 ± 0.39g**
(Figure 12 and Table 3).

Both smoke-exposed groups experienced a significant reduction in food intake
compared to their sham counterparts over the 8-week period (Figure 12C). In addition,
ebselen had no effect on food intake in either the sham or smoke-exposed animals. The
average daily food intake of the animals was 2.66 ± 0.10g, 2.75 ± 0.10g, 2.45 ± 0.09g, and
2.53 ± 0.08g for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen
groups respectively.
Figure 12 The effect of ebselen (10mg kg⁻¹) administration on total body weight and food consumption in mice after smoke exposure.

(A) Body weight was recorded daily for each group over the 56-day period. (B) Final percentage weight change on day 56. (C) Food intake was calculated by measuring the amount of food remaining in the cages and dividing the difference between days by the number of mice in the cage. Data are expressed as mean ± SEM for n=12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

3.3.3. Impact of cigarette smoke exposure on organ and skeletal muscle weight

Lungs

In this study, only the lungs, hind limb skeletal muscles, and WAT were collected. Smoke-exposure caused a significant increase in lung weight, which was not reduced by treatment with ebselen. Specifically, compared to the sham + vehicle control, the smoke + vehicle mice lung weight increased by 15.0% and the smoke + ebselen group by 13.5% (Table 3).
**Skeletal Muscle**

In this study, muscle wasting was observed in the gastrocnemius, plantaris and both RWAT and TWAT. Specifically, cigarette smoke exposure significantly reduced the weights of the gastrocnemius and plantaris by 7%, and 8.3% respectively (Table 3). In contrast to previous studies, significant muscle wasting was not observed in the tibialis anterior and soleus muscles. Treatment with ebselen had no effect on the significant wasting observed in the gastrocnemius but appears to have had a protective effect on the plantaris (Table 3). The tibias of the animals were collected and their length measured. The lengths of the TA and gastrocnemius were normalised to the tibia length, however this did not affect the statistical significance of the muscle wasting observed due to cigarette smoke exposure. This indicates that the wasting observed was not proportional to the observed reduction in total body weight of the cigarette smoke-exposed mice.

**White adipose tissue**

Smoke exposure significantly reduced the weight of the testicular WAT and retroperitoneal WAT by 39.2% and 43.3% respectively (Table 3). As with Study A, treatment with ebselen did not protect mice from this cigarette smoke-induced reduction WAT. Both RWAT and TWAT were collected from this study, however, due to time constraints they were not analysed.
Table 3. The effect of ebselen (10 mg kg⁻¹) administration on lung, skeletal muscle weights, and WAT weights in mice after cigarette smoke exposure.

Data are expressed as mean ± SEM for n=12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sham Vehicle</th>
<th>Sham Ebselen</th>
<th>Cigarette Smoke Vehicle</th>
<th>Cigarette Smoke Ebselen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>24.0 ± 0.28</td>
<td>24.4 ± 0.29*</td>
<td>21.9 ± 0.34*</td>
<td>22.7 ± 0.34*</td>
</tr>
<tr>
<td>Tibialis anterior (mg)</td>
<td>58.4 ± 1.0</td>
<td>54.5 ± 2.4</td>
<td>54.3 ± 0.9</td>
<td>55.4 ± 1.1</td>
</tr>
<tr>
<td>Soleus (mg)</td>
<td>10.3 ± 0.4</td>
<td>9.20 ± 0.4</td>
<td>9.60 ± 0.2</td>
<td>9.30 ± 0.5</td>
</tr>
<tr>
<td>Gastrocnemius (mg)</td>
<td>147 ± 1.6</td>
<td>144 ± 2.6</td>
<td>137 ± 1.6 *</td>
<td>136 ± 2.6 *</td>
</tr>
<tr>
<td>Plantaris (mg)</td>
<td>19.0 ± 0.3</td>
<td>18.6 ± 0.5</td>
<td>17.4 ± 0.3 *</td>
<td>17.6 ± 0.4</td>
</tr>
<tr>
<td>Testicular WAT (mg)</td>
<td>509 ± 27</td>
<td>477 ± 35</td>
<td>310 ± 23 *</td>
<td>322 ± 18 *</td>
</tr>
<tr>
<td>Retroperitoneal WAT (mg)</td>
<td>82.7 ± 7.2</td>
<td>76.8 ± 7.4</td>
<td>46.9 ± 11.4 *</td>
<td>43.7 ± 3.3 *</td>
</tr>
<tr>
<td>Lung (mg)</td>
<td>266 ± 3.3</td>
<td>258 ± 5.9</td>
<td>306 ± 5.3 *</td>
<td>302 ± 9.1 *</td>
</tr>
</tbody>
</table>

3.3.4. Assessment of tibialis anterior muscle function in situ

Neither forelimb nor total grip strength was measured in this study. The maximum force-frequency, rate of contraction, force generated at 120 Hz, and the specific force at 120 Hz were measured using a force transducer in situ.

Force Frequency

As seen in Figure 13A, the frequency-dependent gain in contractile force was observed in the mice, with the peak occurring at approximately 100-120 Hz for the sham + vehicle control group. Following smoke exposure, there were no alterations to the force-frequency relationship in terms of the peak threshold. This indicates that there is no observed muscle impairment in terms of contractility in this study. Surprisingly, the smoke + ebselen treated mice showed the greatest force generated at all frequencies above 30Hz and this is reflected in both the rate of contraction (Figure 13C) and maximum force at 120Hz (Figure 13B).
**Rate of Contraction**

As seen in Figure 13B, the frequency dependent gain in the maximum rate of contraction was observed in all groups. As with the force-frequency (Figure 13A), smoke exposure did not alter the frequency dependent gain. Surprisingly, the sham + vehicle showed a lower maximum rate of contraction at all frequencies compared to every other treatment group. In addition, the smoke + ebselen group showed a trend of increased maximum rates of contraction at all frequencies above 30 Hz, which may be a reflection of the increased force generated at all frequencies as seen in Figure 13A.

**Max Force at 120Hz, Specific Force at 120Hz, and Absolute Max Force**

In this study, peak force generation occurred at 100-120 Hz for all groups. The maximum force at 120Hz did not significantly differ between the 4 groups, regardless of exposure or treatment (Figure 13C). Surprisingly, there was a slight trend for the smoke-exposed groups to have increased force generation at 120 Hz. Specifically, maximum force at 120 Hz was $721 \pm 28.6$ mN, $692 \pm 25.0$ mN, $772 \pm 31.8$ mN, and $829 \pm 36.8$ mN for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen groups respectively.

The maximum force at 120Hz was normalised using the $L_o$, the optimised 100 Hz twitch test length of each TA muscle, which provided the specific force at 120Hz. Specific force was $186 \pm 9.5$ mN, $187 \pm 8.5$ mN, $187 \pm 8.8$ mN, and $189 \pm 10.8$ mN respectively. As with the maximum force, the specific force did not significantly differ between the 4 groups, regardless of exposure or treatment (Figure 13D). In addition, the trend observed in the maximum force (Figure 13C) was ameliorated once the data was normalised. The same result is seen in Figure 13E where there is no difference in the absolute maximum force generated was observed regardless of frequency. Absolute maximum force was $735 \pm 35.1$ mN for the sham + vehicle group and $787 \pm 26.7$ mN for the smoke + vehicle group.
Figure 13. Effect of ebselen (10mg kg⁻¹) administration on tibialis anterior contractile function in mice after cigarette smoke exposure.

(A) Maximum force-frequency relationship, (B) maximum rate of contraction, (C) maximum force generated at 120Hz, (D) specific force generated at 120Hz, and (E) maximum absolute force. Data are expressed as mean ± SEM for n=6-12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance in A-D. Student’s t-test was performed to assess statistical significance in E.
3.3.5. Assessment of mRNA expression in skeletal muscle tissue

Tibialis anterior

In this study, there was no observed wasting in the tibialis anterior muscle. To determine possible causes of the lack of wasting even though there was an abundance of lung inflammation, we measured the mRNA expression of Atrogin-1, MuRF-1, IGF-1 (eA and eB isoforms), myostatin, IL-6, Gpx-1, Ppargc1a and Nfe2l2 in the gastrocnemius via qRT-PCR. Surprisingly, there were no significant differences in expression of any of the genes measured as a result of either cigarette smoke exposure or treatment with ebselen (Table 4).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham Vehicle</th>
<th>Ebselen</th>
<th>Cigarette Smoke Vehicle</th>
<th>Ebselen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>1.00 ± 0.14</td>
<td>1.12 ± 0.15</td>
<td>1.12 ± 0.17</td>
<td>1.20 ± 0.11</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>1.00 ± 0.12</td>
<td>0.83 ± 0.12</td>
<td>0.80 ± 0.09</td>
<td>0.96 ± 0.16</td>
</tr>
<tr>
<td>IGF-1eA</td>
<td>1.00 ± 0.16</td>
<td>0.94 ± 0.15</td>
<td>1.18 ± 0.10</td>
<td>1.09 ± 0.13</td>
</tr>
<tr>
<td>IGF-1eB</td>
<td>1.00 ± 0.14</td>
<td>1.01 ± 0.18</td>
<td>1.19 ± 0.16</td>
<td>1.09 ± 0.09</td>
</tr>
<tr>
<td>Myostatin</td>
<td>1.00 ± 0.17</td>
<td>0.70 ± 0.07</td>
<td>0.86 ± 0.06</td>
<td>0.91 ± 0.10</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00 ± 0.20</td>
<td>0.79 ± 0.08</td>
<td>1.00 ± 0.12</td>
<td>0.80 ± 0.03</td>
</tr>
<tr>
<td>Gpx-1</td>
<td>1.00 ± 0.17</td>
<td>0.97 ± 0.16</td>
<td>1.26 ± 0.29</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>PPARGC1A</td>
<td>1.00 ± 0.13</td>
<td>1.17 ± 0.27</td>
<td>1.00 ± 0.11</td>
<td>0.98 ± 0.13</td>
</tr>
<tr>
<td>Nfe2l2</td>
<td>1.00 ± 0.06</td>
<td>1.30 ± 0.28</td>
<td>1.13 ± 0.10</td>
<td>1.10 ± 0.10</td>
</tr>
</tbody>
</table>

Table 4 The effect of ebselen (10mg kg\(^{-1}\)) administration on tibialis anterior tissue mRNA expression of Atrogin-1, MuRF-1, IGF-1, myostatin, IL-6, Gpx-1, Ppargc1a and Nfe2l2 in mice after cigarette smoke exposure.

Data are expressed as mean ± SEM for n=9-12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance.

Gastrocnemius

In this study, there was significant wasting of the gastrocnemius as a result of cigarette smoke exposure. Although significant improvements in gastrocnemius mass were not observed following treatment with ebselen, significant wasting was still observed in these tissues which warranted analysis to help elucidate the mechanism by which this wasting occurred.
The mRNA expression of Atrogin-1, muscle RING-finger protein-1 (MuRF-1), insulin-like growth factor (IGF-1eA and eB isoforms), myostatin, IL-6, Gpx-1, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) and Nuclear factor, erythroid 2 like 2 (Nfe2l2) in the gastrocnemius was measured via qRT-PCR. As with the tibialis anterior, there were no observed changes to mRNA expression in the gastrocnemius (Table 5).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>Cigarette Smoke</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Ebselen</td>
</tr>
<tr>
<td>Atrogin-1</td>
<td>1.00 ± 0.12</td>
<td>1.07 ± 0.18</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>1.00 ± 0.17</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>IGF-1eA</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>IGF-1eB</td>
<td>1.00 ± 0.14</td>
<td>1.06 ± 0.10</td>
</tr>
<tr>
<td>Myostatin</td>
<td>1.00 ± 0.09</td>
<td>1.13 ± 0.06</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00 ± 0.10</td>
<td>1.17 ± 0.18</td>
</tr>
<tr>
<td>Gpx-1</td>
<td>1.00 ± 0.05</td>
<td>1.01 ± 0.08</td>
</tr>
<tr>
<td>PPARGC1A</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>Nfe2l2</td>
<td>1.00 ± 0.04</td>
<td>0.97 ± 0.04</td>
</tr>
</tbody>
</table>

Table 5. The effect of ebselen (10 mg kg⁻¹) administration on gastrocnemius tissue mRNA expression of Atrogin-1, MuRF-1, IGF-1, myostatin, IL-6, Gpx-1, PPARGC1A and Nfe2l2 in mice after smoke exposure.

Data are expressed as mean ± SEM for n=9-12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance.

3.3.6. Markers of systemic inflammation and oxidative stress

C-reactive protein

C-reactive protein (CRP) is an acute phase protein that is commonly used as a marker of systemic inflammation. CRP is produced in the liver and is measured in serum. In humans, CRP levels can be elevated because of chronic inflammatory conditions such as arthritis, cancer, and COPD, or as a result of acute conditions such as infections, burns and trauma [232, 233].
In this study, cigarette smoke exposure caused a significant increase in serum CRP levels but this was not improved by treatment with ebselen. Compared to the sham + vehicle group, there was an increase of 31.8% in the smoke + vehicle group and 27.9% in the smoke + ebselen group (Figure 14A). Specifically, CRP was $2.55 \times 10^3 \pm 148$ ng/mL, $3.21 \times 10^3 \pm 240$ ng/mL, $3.37 \times 10^3 \pm 171$ ng/mL*, and $3.27 \times 10^3 \pm 147$ ng/mL* for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen groups respectively.

**TBARS (Malondialdehyde)**

As ROS and RNS have extremely short half-lives, they are difficult to measure directly. Consequently, the products of oxidative damage were measured as surrogates of ROS and RNS levels. One type of product of oxidative stress that can be detected in serum is thiobarbituric acid reactive substances (TBARS). A commonly measured TBARS is malondialdehyde (MDA), which is formed as a by-product of lipid peroxidation. The TBARS assay uses thiobarbituric acid as a reagent to measure the concentration of MDA and measures the MDA generated from lipid hydroperoxides by the hydrolytic conditions of the reaction.

In this study, cigarette smoke exposure had no impact on serum MDA levels and, subsequently, neither did treatment with ebselen (Figure 14B). Specifically, MDA was measured as $20.8 \pm 0.57$ ng/mL, $21.8 \pm 1.29$ ng/mL, $19.8 \pm 1.06$ ng/mL*, and $20.8 \pm 0.91$ ng/mL* for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen groups respectively.
Figure 14 The effect of ebselen (10mg kg\(^{-1}\)) administration on markers of systemic inflammation and systemic oxidative stress in mice after cigarette smoke exposure.

(A) Levels of C-reactive protein (CRP), and (B) Malondialdehyde (MDA). Data are expressed as mean ± SEM for n=7-8 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

3.3.7. **Assessment of oxidative stress in gastrocnemius tissue**

As with previous studies, protein was extracted from gastrocnemius tissues and protein carbonylation was measured using an oxyblot chemiluminescence assay as a marker of oxidative stress. Gastrocnemius tissue was used as all the tibialis anterior tissue had been consumed for gene expression analysis. Compared to the sham + vehicle treated group, cigarette smoke exposure caused a significant increase in protein carbonylation which was unaltered by treatment with ebselen (Figure 15A and 15B). This is consistent with the observed changes to gastrocnemius mass where ebselen had no effect on tissue mass. Specifically, protein carbonylation densitometry of the gastrocnemius tissue was measured to be 3.14x10\(^6\)±5.8x10\(^5\)Int, 9.14x10\(^6\)±2.16x10\(^5\)Int, 2.85x10\(^7\)±4.0x10\(^6\)Int*, and 3.78x10\(^7\)±4.6x10\(^6\)Int* for the sham + vehicle, sham + ebselen, smoke + vehicle, and smoke + ebselen groups respectively after normalisation to \(\beta\)-actin (Figure 15B).
Figure 15. The effect of ebselen (10 mg kg\(^{-1}\)) administration on protein carbonylation in gastrocnemius tissue in mice after smoke exposure.

(A) Representative oxyblot showing differences in smearing between treatment groups: Sham + Vehicle (SV), Sham + Ebselen (SE), Cigarette Smoke + Vehicle (CSV) and Cigarette Smoke + Ebselen (CSE). (B) Densitometry analysis of oxyblots normalised to β-actin. Data are expressed as mean ± SEM for n=3-4 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

Due to time constraints, the muscle samples that were collected for histological examination could not be examined. Additionally, the gene expression of the lung samples was not assessed due to the focus of the experiments on the skeletal muscle aspects of the study. This should be conducted in the future to explore the anti-inflammatory effects of ebselen in the lung in further detail.

3.4. Discussion

The principal objective of these studies was to investigate whether reducing levels of H\(_2\)O\(_2\) with the Gpx-1 mimetic ebselen protects against cigarette smoke-induced lung inflammation and skeletal muscle wasting in an established animal model of COPD.
This study showed that mice exposed to sub-chronic cigarette smoke have enhanced BALF inflammation whereas treatment with ebselen attenuated this increase in inflammation (Figure 7 and 11). As seen in previous studies [207], smoke exposed mice, regardless of treatment, had a significant increase in total cells, macrophages and neutrophils in the BALF. In both study A and B, smoke exposed mice treated with ebselen experienced a significant reduction in neutrophil numbers of approximately 50-55% but the treatment did not impact macrophage numbers. This reduction in neutrophils is similar in magnitude to that seen in acute smoke exposure studies. Animals exposed to cigarette smoke, inoculated with influenza A virus and treated with 10 mg/kg of ebselen per day for 7 days experienced a 50% decrease in neutrophil counts in BALF [229]. It should be noted that a slight but significant reduction in macrophages and overall total cell counts in BALF was observed in an acute smoke exposure model as a result of ebselen treatment and this was not observed in Study B. The similarity between the BALF cell counts of the vehicle treated and ebselen treated sham mice indicates that reducing H$_2$O$_2$ with ebselen had no effect on BALF cellularity in the sham-exposed mice in both studies. Due to ebselen’s mechanism of action, we believe that the observed anti-inflammatory effects are a result of reduced ROS leading to reduced inflammation as hypothesised in Chapter 1. The significant increase in lung mass observed in all smoke exposure groups can be attributed to increased inflammation and pulmonary oedema (Table 2 and 3). It has been shown previously that 8 weeks of smoke exposure does not induce changes in airspace in BALB/c mice [234] which typically takes a much longer period of time (6 months) or through the use of a nose-cone smoke exposure model [235, 236], therefore, airspace enlargement as a model of emphysema was not determined in the current study.

Consistent with previous studies conducted by our group, cigarette smoke exposure increased mRNA expression of a number of inflammatory cytokines and chemokines in the
lungs of the mice (Figure 8). It should be noted that lung mRNA expression was not assessed in Study B due to time limitations. The pro-inflammatory cytokines TGF-β, IL-1β and IL-6 are known drivers of pathology in chronic inflammatory diseases and the concentration of these cytokines is elevated in the serum and lungs of COPD patients [237, 238]. In this model, smoke exposure caused an increase in expression of these pro-inflammatory cytokines, which was attenuated by treatment with ebselen. This indicates that the increased reduction of hydrogen peroxide Gpx-1 mimetic ebselen may have therapeutic benefit for the treatment of pulmonary manifestations of COPD.

MCP-1 and CXCL-10 are important monocyte recruitment factors responsible for macrophage accumulation [239, 240]. These chemoattractants are increased in the lungs of patients with both chronic and acute lung inflammation and increased expression of these genes has been observed in various respiratory conditions, including COPD [238, 241, 242]. The increase in MCP-1 and CXCL-10 gene expression in lung tissue of smoke-exposed vehicle treated mice most likely explains the very large increase in BALF macrophage numbers (Figure 8B and 8E). While there was an increase in the expression of both MCP-1 and CXCL10 in the lungs as a result of smoke exposure, treatment with ebselen only reduced expression of MCP-1. As there was no significant reduction in macrophage numbers in BALF as a result of treatment with ebselen, even though MCP-1 expression was reduced, indicates that CXCL10 may be playing a greater role in macrophage chemoattraction in this model.

MIP-2α is a chemokine, secreted by macrophages, which acts as a polymorphonuclear leukocyte chemoattractant, and IL-17A is a cytokine involved in activating neutrophil-mobilizing factors such as IL-8 and GM-CSF and hence sustaining the accumulation and activity of neutrophils locally in the lungs [243]. The increase in expression of MIP-2α, IL-17A and, subsequently, GM-CSF may all be contributing to the increase in
neutrophils observed in the BALF of the smoke exposed animals (Figure 8A, 8C and 8K). Additionally, treatment with ebselen caused a marked reduction in both the expression of these genes and the number of neutrophils in BALF, further emphasizing that MIP-2α, IL-17A and GM-CSF are driving neutrophil accumulation in this smoke model. Another potential explanation of the high macrophage counts and decreased neutrophil counts is that the macrophages may be clearing the neutrophils in the lungs via efferocytosis, however, further experiments are required to determine the exact mechanism of this phenomenon.

The expression of MMP9, was also increased by smoke exposure but was not reduced by treatment with ebselen (Figure 8H). The MMP family of enzymes are involved in the breakdown of extracellular matrix in normal physiological processes but are also implicated in pathological processes, such as in inflammatory diseases [244]. It should be noted that MMP9 in particular appears to be a regulatory factor in neutrophil migration in addition to its proteolytic functions [245]. While this combination of neutrophil chemoattraction and extracellular degradation makes MMP9 a potential key driver of lung inflammation, it does not appear to be a key driver of disease in this model.

In addition to pulmonary inflammation that is characteristic of the disease, COPD patients also have elevated levels of various biomarkers indicating the presence of systemic inflammation [246, 247]. Systemic inflammation is associated with an accelerated decline in lung function [248] and is also a driver of systemic oxidative stress, which in turn further perpetuates systemic inflammation [102]. This vicious cycle leads to systemic manifestations of disease in the form of comorbidities, such as peripheral skeletal muscle wasting [112]. Serum concentration of CRP, an acute phase protein that is reported to be elevated in COPD patients, was measured as a marker of systemic inflammation in this model. As expected, serum CRP levels were elevated in response to smoke exposure, however, treatment with ebselen had no effect (Figure 14A). This is in contrast to the reduced BALF cellularity
observed in the treatment animals. This indicates that although lung inflammation is hypothesized to spillover into systemic circulation in COPD [249], this phenomenon may not be a key driver of systemic inflammation in this model and, subsequently, not a key driver of systemic manifestations of disease either. This indicates that the observed systemic inflammation is likely originating outside of the lungs. This is consistent with the hypothesis that systemic inflammation in COPD is not necessarily related to local pulmonary inflammation but instead originates from a parallel inflammatory response in other tissues, such as adipose tissue [250, 251]. It should be noted that significant systemic oxidative stress, as measured by MDA concentration in serum, was not observed in response to either smoke exposure and, therefore, the impact of ebselen on systemic oxidative stress could not be determined in this study (Figure 14B). This mild systemic response to smoke exposure was further emphasized by the modest impact that smoke exposure had on skeletal muscle mass.

Both acute and chronic smoke exposure is known to reduce total body mass in animal models [252, 253]. This smoke-induced reduction in body weight is mostly caused by a reduction in adipose tissue; however, reduced muscle tissue mass also contributes to this decrease. As expected, the smoke exposed animals experienced a significant reduction in total body weight compared to their sham exposed counterparts (Figure 9A and 9B). Ebselen did not have a significant effect against smoke induced weight loss. Additionally, smoke exposure caused a significant reduction in adipose tissue mass and this also was not improved by treatment with ebselen (Tables 2 and 3). This indicates that the beneficial effect that ebselen demonstrated in the lungs may not extend to systemic pathologies.

While the appetite suppressing effect of cigarette smoke is well established [254] and may possibly account for the reduction in body weight and skeletal muscle mass, it has been previously shown by using long-term pair-feeding that cigarette smoke exposure causes a greater reduction in body weight compared to equivalent food restriction alone (smoke
exposure protocols of 1 and 3 months) [147, 207]. In the present study, the smoke-exposed animals had a suppressed appetite that was unaffected by treatment with ebselen (Figure 9C). Additionally, ebselen did not impact the appetite of sham-exposed animals, indicating that any alterations to pathology and physiology of the animals cannot be attributed to increased food intake/caloric surplus in the treatment groups.

Although overall body mass was reduced due to smoke exposure, lung mass was significantly increased as expected. Surprisingly, cigarette smoke caused a significant reduction in heart mass that was not improved by ebselen (Tables 2 and 3). This is in contrast to the hypertrophy of cardiac tissue widely observed in the hearts and vasculature of COPD patients that arises from cardiovascular manifestations of disease [255, 256]. Potential mechanisms driving this reduction in heart mass were explored and are described in chapter 4.

Peripheral skeletal muscle wasting is highly prevalent in COPD and is associated with reduced quality of life, decline in lung function and increased mortality [112, 257]. Increased inflammation and oxidative stress both contribute to the onset and development of skeletal muscle wasting [212, 258]. The targeting of oxidant-dependent mechanisms that overlap between COPD and comorbid muscle wasting should be able to reduce oxidative stress and subsequently reduce perpetuation of inflammation, resulting in improved pulmonary symptoms and increased muscle mass and function. It has been reported that smoke exposure is known to cause a reduction in muscle mass of hind limb muscles in mice [207, 259]. In this study, smoke exposure caused a significant reduction in mass of the larger hind limb muscles, specifically the tibialis anterior and the gastrocnemius (Tables 2 and 3). It should be noted that TA wasting was only observed in study A. Although previously reported [259-261], wasting of the smaller gastrocnemius complex muscles (the soleus and plantaris) was not observed in this model. While the lack of sensitivity of the scales used in this study may have
hindered the ability to measure slight reductions in mass of the soleus and plantaris, it is more likely that the response to smoke was too mild to promote significant wasting in these tissues. Treatment with ebselen did not protect against smoke induced muscle wasting even though lung pathology was significantly improved. These results are concordant with the changes observed in body weight, white adipose tissue, systemic inflammation and systemic oxidative stress. This further indicates that the pulmonary response to cigarette smoke exposure was not driving the systemic pathologies observed in this model.

Skeletal muscle dysfunction commonly accompanies skeletal muscle wasting in COPD [262]. Occasionally, this dysfunction can be disproportional to the muscle mass loss; however this is only observed in patients chronically treated with glucocorticosteroids [112]. Furthermore, muscle dysfunction in COPD is also associated with a significantly poorer prognosis and increased mortality [263]. Despite the fact that COPD patients experience skeletal muscle dysfunction in both respiratory and peripheral muscle groups, the latter are generally affected with greater severity [264, 265]. In patients, muscle function is commonly measured by quadriceps strength and endurance since the upper limb muscles are frequently spared from severe wasting [18, 120, 266-268]. Due to anatomical differences, muscle function in mouse models is usually measured via whole limb function (grip strength testing) or individual hind limb muscle contractility [269, 270]. In Study A, both total limb and forelimb grip strength was assessed by means of a commercially available automatic grip strength meter. Cigarette smoke caused a significant decrease in both total limb and forelimb grip strength that was unaffected by ebselen (Figure 10A and 10B). When normalized to body weight, this reduction is no longer observed, indicating that the dysfunction is proportional to total body mass (Figure 10C and 10D). This is in accordance with the proportional loss in quadriceps muscle mass and function observed in COPD patients [271]. Due to the greater capacity for human error when assessing function using grip strength
meters, isolated whole muscle contractility was used to assess TA muscle function in Study B. In contrast to the grip strength results from Study A, no significant reduction in muscle contractility, endurance or force generation was observed in response to smoke exposure in Study B, although there was a trend towards altered contractile function (Figure 13A and 13C). This insignificant response was unsurprising, as the TA muscle did not experience a significant reduction in mass. Because significant muscle dysfunction was not observed via either assessment, the effect that ebselen has on muscle dysfunction could not be determined.

The finely balanced processes that regulate muscle mass homeostasis can be distinguished into atrophy-related (protein degradation) and hypertrophy-related (protein synthesis) pathways. In skeletal muscle wasting, there is an imbalance between these pathways that shifts the homeostatic balance in favor of protein degradation [272]. This imbalance arises from increased atrophy signaling, decreased hypertrophy signaling, or a combination of the two. The ubiquitin-proteasome system is one of the major atrophy-related pathways in skeletal muscle and is widely studied in cachexia, sarcopenia and comorbid skeletal muscle wasting [273]. Atrogin-1 and MuRF-1 are E3 ubiquitin ligases that are key regulators of ubiquitin-mediated protein degradation in skeletal muscle [274, 275]. Increased transcription of these ligases results in downstream increases in protein degradation and drives atrophy of the muscle if chronically elevated. This is evident in biopsies taken from the quadriceps of COPD patients [276, 277]. In this study, smoke exposure had no significant effects on Atrogin-1 or MuRF-1 mRNA expression in the tibialis anterior and gastrocnemius muscles (Table 4 and 5). Additionally, smoke exposure had no effect on mRNA levels of other proteins involved in atrophy or hypertrophy related pathways, specifically myostatin and IGF-1. This indicates that the reduction in muscle mass was not driven by the characteristic alterations to protein homeostasis signaling pathways observed in COPD patients.
Additionally, the expression of IL-6 was measured as an indicator of local skeletal muscle inflammation and was also found to be unaffected by smoke exposure. This indicates that while there was a significant increase in pulmonary inflammation in the smoke exposed mice, the significant reduction in skeletal muscle mass was not being driven by a similar local inflammatory response in the muscles. In addition to reduced muscle mass, metabolic alterations in muscle such as reduced oxidative enzyme activity and reduced mitochondrial function are known contributors to skeletal muscle weakness and fatigue in COPD [278-280]. The mRNA expression of Nfe2l2 and PARGC1A, which regulate mitochondrial function and mitochondrial biogenesis respectively [281, 282], was measured to assess mitochondrial impairment following smoke exposure and no significant alterations to mRNA expression were found. This was expected as muscle function was not impaired in response to smoke exposure. Lastly, while the mRNA expression of other glutathione-related enzymes is known to acutely increase following exposure to cigarette smoke, Gpx-1 expression does not increase [283, 284]. This phenomenon was also observed in this study, as Gpx-1 expression was unchanged in the gastrocnemius of the smoke exposed animals.

While there is no strong relationship between muscle oxidative stress and local inflammation in patients with COPD [101, 285], 8 weeks of smoke exposure induced a significant increase in skeletal muscle oxidative stress in this model as measured by protein carbonylation in the gastrocnemius (Figure 15A and 15B). These oxidative alterations were not accompanied by a marked increase in local inflammation (as measured by IL-6 mRNA expression), which echoes what is seen in COPD. Evidence of strong oxidative stress is consistently found in peripheral muscles even if levels of local and systemic levels of inflammatory mediators are relatively low in these patients [101, 286, 287]. While inflammation and oxidative stress both initiate the proteolysis cascade, increased oxidative stress is also known to impair contractility in peripheral skeletal muscles [288, 289].
Although muscle contractility was unaffected in this study, there was a significant increase in protein carbonylation following smoke exposure, which was unaffected by treatment with ebselen (Figure 15B). This indicates that while ebselen was able to improve pulmonary inflammation, it was ineffective in preventing oxidative damage in skeletal muscle.

In summary, the findings of this study identified mimicking GPX-1 activity, via treatment with ebselen, to be an effective therapy for pulmonary inflammation in this model of COPD. Ebselen successfully reduced smoke-induced increases in lung inflammation, lung inflammatory cytokine and chemokine mRNA expression. The therapeutic impact of ebselen on systemic manifestations of disease was not determined in this study. Ebselen had no effect on smoke-induced skeletal muscle and WAT wasting, expression of muscle growth signaling pathways, muscle contractility and function. This study shows that while smoke exposure induced the pulmonary and some of the systemic alterations characteristic of COPD, ebselen was only able to effectively treat the pulmonary manifestations of disease in this model.
Chapter 4: The effect of apocynin in an 8-week CS-exposure *in vivo* model of COPD
4. Introduction

In this chapter, the effect that treatment with apocynin had on lung inflammation and skeletal muscle wasting in an 8-week smoke exposure model of COPD is explored and all the experimental results are presented and examined in detail. Due to insufficient animal samples, the smoke exposure portion of the study was replicated, however this was completed at another animal facility. We determined that the data sets could not be combined due to variability between the animal facilities (refer to Chapter 6). As this study was replicated, the first study will be referred to as Study A, and the second referred to as Study B for the purpose of this thesis.

COPD is characterized by persistent airflow limitation and lung inflammation resulting in a progressive decline in lung function [12]. In addition to the pulmonary manifestation of COPD, many systemic manifestations occur in the form of comorbid diseases, such as skeletal muscle wasting [13-15]. Skeletal muscle wasting occurs in approximately 20 to 40% of all COPD patients [17, 111, 112] and is multifactorial in nature. Such factors include inflammation and oxidative stress and these factors can initiate or enhance negative alterations in peripheral muscles of patients with COPD [112]. Chronic oxidative stress not only contributes to muscle wasting, but it is also associated with dysfunction in both respiratory and peripheral skeletal muscle.

Aside from exercise therapy, there are currently no effective treatments for skeletal muscle wasting in COPD. Pharmacological treatments that have been explored include, anabolic steroids, growth hormone, other anabolic compounds, bioactive nutrients (e.g. ghrelin, creatine) and antioxidants [212-214]. Most of these treatments have only shown little to modest benefits when treating skeletal muscle wasting and weakness in COPD [215-217]. Therefore, new treatments are needed, as limiting muscle wasting in COPD will lead to
improved quality of life and increased survivability of patients. Because of its role in the pathogenesis of comorbid skeletal muscle wasting, targeting oxidative stress may be able to slow the progression or onset of the disease, giving patients an improved quality of life while managing their other symptoms.

As described in chapter 1, nicotinamide adenine dinucleotide phosphate oxidase, or NADPH oxidase (NOX) -1 and -2 are the primary generators of O$_2^-$ and are responsible for the initiation of the ROS cascade. NOX-2 is a unique target in the sense that inhibiting the activity of the enzyme would not only reduce the generation of a single oxidant species but also reduce the generation of all other ROS and RNS. NOX-2 is an enzyme complex, consisting of p47phox and p67phox subunits, membrane-bound cytochrome b558, p22phox, and a small G protein ‘rac’ [290, 291]. Following the assembly of the subunits, the enzyme activates and generates superoxide via the one-electron reduction of molecular oxygen [292].

It is well established that increased NOX-2 activity leads to oxidative damage and can contribute to the development oxidative stress under inflammatory conditions [293-295]. Apocynin, also known as acetovanillone, is an organic compound that inhibits the release of O$_2^-$ by NOX. Apocynin blocks the migration of the p47phox subunit to the membrane, preventing the assembly of the functional NOX complex, which then leaves the enzyme in an inactive state. Apocynin administered locally by nebulization has been shown to be safe and reduces ROS concentrations in healthy subjects and mild asthmatics [296, 297]. Apocynin has been shown to suppress inflammation in several pre-clinical animal models and clinically, COPD patients treated with apocynin experienced a reduction in H$_2$O$_2$ and NO$_2^-$ in their exhaled breath concentrate compared to placebo control [201, 298]. Although not widely studied in chronic inflammatory conditions, apocynin’s anti-inflammatory and antioxidant effects make it a key candidate for the treatment of COPD and its comorbidities.
The aim of this study was to investigate whether the NOX-2 inhibitor apocynin protects against cigarette smoke-induced lung inflammation and skeletal muscle wasting in an established subchronic preclinical model of COPD.

4.1. Materials and Methods

4.1.1. Animals

Eight-week old male Balb/c mice were obtained from the Animal Resource Centre Pty. Ltd. (Perth, WA) and housed at 20°C in sterile micro-isolator cages under a 12:12h light dark cycle. Mice were given ad libitum access to food and water. Experimental protocols and procedures were approved by the Animal Ethics Committee of the University of Melbourne (AEC 1112185, 1413348), and the RMIT Animal Ethics Committee (AEC 1521).

4.1.2. Cigarette Smoke Exposure

Mice were exposed to the smoke produced by 3 cigarettes (Winfield Red; Philip Morris, Melbourne, Australia), 2 times a day, 5 days per week over a 56-day period as described in section 2.2.3.

4.1.3. Apocynin Administration

Mice were treated with apocynin (5 mg/kg; Sigma-Aldrich, Sydney, Australia) or vehicle (0.1% DMSO in Saline) via intraperitoneal (i.p) injection 1 hour prior to their first cigarette smoke exposure of the day. Further details on the experimental design and how apocynin was prepared can be found in sections 2.2.2 and 2.2.4.

4.1.4. Body Weight and Food Consumption

Body weight was measured 3 times each week (Monday, Wednesday, Friday) at approximately the same time each weighing day. Food consumption was calculated by
measuring the amount of food remaining in cages every 24 hours and dividing that by the number of mice in the cages.

4.1.5. Muscle Function

The force-frequency relationship of the tibialis anterior muscle was measured in situ using the 1300A: 3-in-1 Whole Animal System – Mouse (Aurora Scientific, Canada). Frequencies ranging from 10Hz to 250Hz were used and further details can be found in section 2.8.1.

4.1.6. Blood, BALF and Tissue Collection

On day 56 mice were killed by an overdose of anaesthetic (pentobarbitone, 200mg/kg prepared in sterile saline), blood collected from the abdominal vena cava, transferred into a Microvette® collection tube coated with lithium heparin (Sarstedt, Germany), and centrifuged at 10,000 x g rpm for 5 min to separate the plasma. Plasma was stored at -80 °C for later analysis. Bronchoalveolar lavage fluid (BALF) was collected from each mouse by flushing with a 400 µL aliquot of PBS, followed by three 300 µL aliquots of PBS as described in section 2.3. The thymus, spleen, and kidneys were removed and weighed. The lungs, the left side hind limb skeletal muscles (tibialis anterior, soleus, plantaris, gastrocnemius), testicular white adipose tissue (WAT) and retroperitoneal WAT were also removed, weighed, snap-frozen in liquid nitrogen and stored at -80 °C for subsequent analysis. The right side hind limb skeletal muscles (tibialis anterior, soleus, plantaris, gastrocnemius) were removed, weighed, embedded in OCT and frozen in melting isopentane, and stored at -80 °C for subsequent analysis. The liver was also removed and weighed, with the largest lobe snap-frozen and stored at -80 °C for subsequent analysis. The total number of viable cells, macrophages, neutrophils and lymphocytes in the BALF was determined as described in section 2.4.
4.1.7. Quantitative RT-qPCR

In the lungs, the mRNA expression of the following inflammatory cytokines, proteins and enzymes was measured: MMP-9, MMP-12, GM-CSF, G-CSF, TNF-α, CXCL9, IL-6, IL-23, IL-17, IL-1β, MCP-1, and MIP-2α. In the tibialis anterior and gastrocnemius muscles, the mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 were measured. For the in vitro C2C12 mouse skeletal muscle cells, IL-6 and TNF-α were measured. GAPDH was used as the reference gene against all genes measured and all measurements were made using commercially available, validated Taqman primer/probes. In order to measure as many genes as possible while rationing collected samples, preliminary analysis used pooled samples from each animal in each group. As the samples were pooled, only trends could be observed and statistical significance could not be determined. Refer to section 2.7 for RNA extraction and RT-qPCR protocol information.

4.1.1. ELISAs and TBARS

The protein concentrations of IL-6, IGF-1, Mouse CRP and 8-isoprostane in serum were determined using commercially available ELISA kits and measured according to manufacturer’s instructions and as detailed in section 2.5. The concentration of MDA was measured using an OxiSelect™ TBARS Assay Kit (Cell Biolabs, Inc., CA, USA) according to manufacturer’s instructions.
4.1.2. Oxyblot

Protein carbonylation, a marker of oxidative stress, was measured in muscle tissue using an Oxyblot Protein Oxidation Detection kit (Merck, Massachusetts, USA). β-actin was used as the reference protein. For further details, refer to sections 2.6.1 and 2.6.2 respectively.

4.1.3. C2C12 Mouse Skeletal Muscle Cells

The C2C12 cell line is a subclone (produced by H. Blau, et al. [299]) of the mouse myoblast cell line established by D. Yaffe and O. Saxel [300]. This cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. Initial pilot studies to highlight the direct impact of cigarette smoke and, more specifically, oxidative stress were conducted using this cell line.

C2C12 myoblasts were cultured in growth media, which consists of Dulbecco’s Modified Eagle Medium (DMEM) with 4.5mmol glucose, glutamine, sodium pyruvate and supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. Seeded myoblasts were differentiated to myotubes using DMEM with 4.5mmol glucose, glutamine, sodium pyruvate supplemented with 2% horse serum and 1% penicillin and streptomycin and as described in section 2.9.3.

4.1.4. H₂O₂ and Cigarette Smoke Extract Exposure

Please refer to sections 2.9.4 and 2.9.5 for relevant protocols.

4.1.5. Cell Viability

C2C12 cell viability following H₂O₂ exposure was measured using commercially available MTT and MTS cell viability assays. Further details can be found in section 2.9.7 and 2.9.8.
4.1.6. Mitochondrial Stress Test

Following H$_2$O$_2$ exposure, C2C12 basal respiration, ATP production, proton leak, maximal respiration, and reserve capacity was measured using the ‘Seahorse’ XF Mito Stress Test Kit (Agilent, CA, USA) according to manufacturer’s specifications. Further details can be found in section 2.9.9.

4.1.7. Statistical Analyses

As data were normally distributed, they are presented as grouped data expressed as mean ± SEM; n represents the number of mice. Student’s t test was used to assess significance where only two data sets were present. Multivariate analysis was performed via one- or two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons, where appropriate. All statistical analyses were performed using GraphPad Prism for Windows and Mac (version 6.0-8.1). In all cases, probability levels less than 0.05 (P < 0.05) were taken to indicate statistical significance.

4.2. Apocynin Study A

4.2.1. Assessment of BALF inflammation

As a measure of local inflammation in the lungs, BALF was collected and total cells were counted. A differential cell count was then performed to determine the number of macrophages and neutrophils. Cigarette smoke exposure (6 cigarettes/day, 5 days/week for 8 weeks) significantly increased the total, macrophage and neutrophil number in BALF (Figure 16) as observed in Chapter 3. However, smoke-exposed mice treated with apocynin had a significant reduction in total cells, macrophages and neutrophils compared with vehicle treated smoke-exposed mice. Following the 56-day cigarette smoke exposure period, the number of total cells in the BALF was 2.48×10$^5$ ± 2.45×10$^4$, 2.74×10$^5$ ± 1.92 ×10$^4$, 9.11×10$^5$ ± 6.72×10$^4$, and 6.53×10$^5$ ± 4.62×10$^4$ cells/mL for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin respectively (Figure 16A). The number of macrophage cells
was $2.48 \times 10^5 \pm 2.46 \times 10^4$, $2.74 \times 10^5 \pm 1.92 \times 10^4$, $6.51 \times 10^5 \pm 4.96 \times 10^4$, and $5.24 \times 10^5 \pm 4.23 \times 10^4$ cells/mL (Figure 16B), whilst the number of neutrophils was $861 \pm 50.6$, $296 \pm 15.4$, $256 \times 10^5 \pm 2.55 \times 10^4$, and $1.28 \times 10^5 \pm 8.99 \times 10^3$ cells/mL for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin respectively (Figure 16C).

![Graph showing total cells, macrophages, and neutrophils in BALF for sham and smoke groups.]

Figure 16. The effect of apocynin (5 mg kg$^{-1}$) administration on BALF cellularity in mice exposed to air (sham) or cigarette smoke (Smoke).

BALF cellularity is shown as (A) the total number of cells, (B) macrophages, and (C) neutrophils. Data are expressed as mean ± SEM for n=8 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.
4.2.2. Assessment of cytokine mRNA expression in lungs

To measure the mRNA expression of inflammatory cytokines and chemokines, lung tissue was pooled and RNA was extracted. Specifically, RT-qPCR was used to measure the expression of macrophage chemoattractants CXCL10, and MCP-1, and neutrophil chemokines MIP-2α, and IL-17A. Cigarette smoke exposure elevated the mRNA expression of CXCL10, MCP-1, MIP-2α, and IL-17A in the lung tissue in the vehicle-treated mice. However, treatment with apocynin caused a reduction in cigarette smoke-induced increases in mRNA expression of CXCL10 and MCP-1 but not MIP-2α and IL-17A. With regards to CXCL10 mRNA expression, compared to the sham + vehicle groups, there was a fold-change of 0.8, 1.6, and 0.7 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 17A). For MCP-1, there was a fold-change of 0.4, 5.1, and 1.8 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 17B). For MIP-2α, there was a fold-change of 0.3, 2.2, and 3.3 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 17C). For IL-17A, there was a fold-change of 0.3, 6.4, and 7.5 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 17D). It was interesting to note that apocynin treatment of sham-exposed mice also resulted in a reduction in CXCL10, MCP-1, MIP-2α and IL-17A (Figure 17 A-D). Due to the pooling of samples, statistical analysis could not be performed for this data.
Figure 17. The effect of apocynin (5 mg kg\(^{-1}\)) on pooled whole lung tissue mRNA expression of cytokines and chemokines in mice exposed to air (sham) or cigarette smoke (smoke).

Data are expressed as mean, n=8 mice per treatment group. As the samples were pooled, this data could not be statistically analysed.
4.2.3. Impact of smoke exposure on total body weight and food consumption

To determine the impact of smoke exposure on weight loss and growth, mouse body weight was measured each day and food consumption was measured on Monday, Wednesday and Friday, with both measurements conducted at approximately the same time per day. Smoke-exposed mice, regardless of treatment, experienced reduced growth compared to their sham counterparts. However, smoke-exposed mice treated with apocynin experienced an improved rate of growth over the vehicle treated group. Figure 18A shows the steady increase in body weights of the mice over the 56-day period. At the end of the 56 day exposure period, the average body weights of the mice increased by 14.0 ± 1.3%, 13.6 ± 1.2%, -0.01 ± 1.5%, and 4.58 ± 1.7% in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively to 25.2 ± 0.49g, 25.2 ± 0.58g, 22.3 ± 0.63g, and 23.0 ± 0.53g (Table 6 and Figure 18B). This is reflected in the final percentage change shown in Figure 18B, which shows that the smoke + apocynin mice had a 32.6% improvement in total body weight over the smoke + vehicle group on day 56 (when compared to the sham + vehicle).

Food intake was measured to determine if apocynin had an impact on nicotine-induced reductions in appetite. As expected, both smoke-exposed groups experienced a significant reduction in food intake compared to their sham counterparts (Figure 18C). In addition, apocynin did not impact daily food intake. The average daily food intake of the animals was 3.71 ± 0.08 g, 3.56 ± 0.07 g, 3.08 ± 0.08 g, and 2.86 ± 0.07 g for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively.
**Figure 18.** The effect of apocynin administration (5 mg kg\(^{-1}\)) on total body weight and food consumption in mice after cigarette smoke exposure.

(A) Body weight was recorded daily for each group over the 56-day period. (B) Final percentage weight change on day 56. (C) Food intake was calculated by measuring the amount of food remaining in the cages and dividing the difference between days by the number of mice in the cage. Data are expressed as mean ± SEM for n=8 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.

**4.2.4. Impact of smoke on organ, skeletal muscle and adipose tissue mass**

To measure the impact of cigarette smoke on skeletal muscle mass, the tibialis anterior, gastrocnemius, plantaris, and soleus were extracted and weighed (Table 6). Cigarette smoke exposure caused a significant reduction in mass of the tibialis anterior and soleus muscles by 8.2 and 7.7% respectively, with apocynin treatment protecting against these changes. Specifically, tibialis anterior mass was 51.2 ± 1.2 mg, 51.8 ± 1.1 mg, 47.0 ± 1.0 mg*, and 48.6 ± 0.99 mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin treatment groups, respectively. The soleus muscle mass followed a similar trend, with a significant reduction in mass upon smoke exposure, which was attenuated by apocynin administration.
apocynin groups respectively. Soleus mass was $7.32 \pm 0.77$ mg, $7.66 \pm 0.87$ mg, $6.76 \pm 0.80^*$, and $7.16 \pm 0.65$ mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively. Surprisingly, the masses of the gastrocnemius and plantaris muscles were not affected by either smoke exposure or apocynin treatment. Specifically, gastrocnemius mass was $123 \pm 3.4$ mg, $124 \pm 2.4$ mg, $116 \pm 2.6$ mg, and $119 \pm 2.5$ mg, and plantaris mass was $15.8 \pm 0.5$ mg, $16.2 \pm 0.4$ mg, $15.3 \pm 0.3$ mg, and $15.0 \pm 0.4$ mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively. Additionally, muscle weight was not normalised to tibia length in this study.

To determine the extent that fat loss contributed to the smoke-induced total body weight loss, testicular and retroperitoneal WAT were extracted and weighed (Table 1). Cigarette smoke caused a significant reduction in the testicular WAT and retroperitoneal WAT by 29.6% and 37.9% respectively, and treatment with apocynin protected against these changes. Specifically, testicular WAT mass was $596 \pm 52$ mg, $534 \pm 66$ mg, $420 \pm 46$ mg*, and $513.9 \pm 41$ mg, and retroperitoneal WAT mass was $106.5 \pm 8.0$ mg, $100.9 \pm 15$ mg, $66.15 \pm 7.2$ mg*, and $89.0 \pm 10^4$ mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively. Unfortunately, the WAT samples were not collected so no further analysis was conducted using these samples.

To measure potential inflammation and oedema in organs, the weights of the lungs, heart, kidney, liver, and spleen were measured (Table 6). Smoke-exposure caused a significant increase in lung weight, which was not reduced by treatment with apocynin. Compared to the sham + vehicle control, the smoke + vehicle mice lung weight increased by 23.7% and the smoke + apocynin group by 30.0%. Specifically, the lung weights were $228 \pm 8.0$ mg, $247 \pm 6.3$ mg, $282 \pm 17.9$ mg, and $296 \pm 16.0$ mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively.
Smoke-exposure significantly reduced heart weight, which was not improved by treatment with apocynin. Compared to the sham + vehicle control, the smoke + vehicle mice heart weight decreased by 8.9% and the smoke + apocynin group by 8.2%. This is in contrast to the expected effect of smoke-exposure causing an increase in cardiac mass. Specifically, the heart weights were 126 ± 3.1 mg, 127 ± 3.1 mg, 115 ± 4.6 mg, and 115 ± 3.8 mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively.

Additionally, smoke-exposure significantly reduced spleen weight, however, the significant reduction was not observed in the apocynin treated mice. Compared to the sham + vehicle control, the smoke + vehicle mice spleen weight decreased by 21.38% and the smoke + apocynin group by 16.2%. Specifically, the spleen weights were 88.8 ± 3.2 mg, 95.7 ± 2.1 mg, 69.8 ± 6.6 mg, and 74.4 ± 2.0 mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively. Spleen tissue was not collected so further analysis could not be conducted.

In general, neither smoke-exposure nor treatment with apocynin had an effect on liver and kidney weights in this model. Specifically, the liver weights were 1.3110^3 ± 78.1 mg, 1.10x10^3 ± 39.8 mg, 1.08x10^3 ± 37.0 mg, and 983 ± 41.0 mg, and the kidney weights were 369 ± 12.4 mg, 372 ± 8.0 mg, 350 ± 14.1 mg, and 341 ± 15.7 mg in the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>SV</th>
<th>SA</th>
<th>CSV</th>
<th>CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>25.2 ± 0.49</td>
<td>25.2 ± 0.58</td>
<td>22.3 ± 0.63 *</td>
<td>230 ± 0.53 *#</td>
</tr>
<tr>
<td>Tibialis anterior (mg)</td>
<td>51.2 ± 1.2</td>
<td>51.8 ± 1.1</td>
<td>47.0 ± 1.0 *</td>
<td>48.6 ± 0.99</td>
</tr>
<tr>
<td>Soleus (mg)</td>
<td>7.32 ± 0.77</td>
<td>7.66 ± 0.87</td>
<td>6.76 ± 0.80 *</td>
<td>7.16 ± 0.65</td>
</tr>
<tr>
<td>Gastrocnemius (mg)</td>
<td>123 ± 3.4</td>
<td>124 ± 2.4</td>
<td>116 ± 2.6</td>
<td>119 ± 2.5</td>
</tr>
<tr>
<td>Plantaris (mg)</td>
<td>15.8 ± 0.5</td>
<td>16.2 ± 0.4</td>
<td>15.3 ± 0.3</td>
<td>15.0 ± 0.4</td>
</tr>
<tr>
<td>Testicular WAT (mg)</td>
<td>596 ± 52</td>
<td>533 ± 66</td>
<td>420 ± 46 *</td>
<td>514 ± 41</td>
</tr>
<tr>
<td>Retroperitoneal WAT (mg)</td>
<td>107 ± 8.0</td>
<td>101 ± 15</td>
<td>66.2 ± 7.2 *</td>
<td>89.0 ± 10 #</td>
</tr>
<tr>
<td>Lung (mg)</td>
<td>228 ± 8.0</td>
<td>247 ± 6.3</td>
<td>282 ± 17.9 *</td>
<td>296 ± 16.0 *</td>
</tr>
<tr>
<td>Heart(mg)</td>
<td>126 ± 3.1</td>
<td>127 ± 3.1</td>
<td>114.6 ± 4.6 *</td>
<td>115.4 ± 3.8 *</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>88.8 ± 3.2</td>
<td>95.68 ± 2.1</td>
<td>69.8 ± 6.6 *</td>
<td>74.4 ± 2.0</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>1.31x10^3 ± 78</td>
<td>1.10x10^3 ± 40</td>
<td>1.08x10^3 ± 37</td>
<td>983 ± 41</td>
</tr>
<tr>
<td>Kidney(mg)</td>
<td>369 ± 12.4</td>
<td>372 ± 8.0</td>
<td>350 ± 14.1</td>
<td>341 ± 15.7</td>
</tr>
</tbody>
</table>

Table 6. The effect of apocynin (5 mg kg⁻¹) administration on lung, heart, kidney, liver, spleen, skeletal muscle weights, and WAT weights in mice after smoke exposure.

Sham + vehicle (SV), sham + apocynin (SA), cigarette smoke + vehicle (CSV), and cigarette smoke + apocynin (CSA) data are expressed as mean ± SEM for n=8 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.

4.2.5. Assessment of mRNA expression in tibialis anterior

Tibialis anterior tissue was pooled and RNA was extracted. The expression of MuRF-1, atrogin-1, IGF-1b, and myostatin was measured via qRT-PCR. Smoke exposure caused a reduction in atrogin-1 mRNA expression but surprisingly, had no effect on MuRF-1 mRNA expression in the tibialis anterior muscle in either treatment group (Figure 19A and 19B). Cigarette smoke caused a reduction in IGF-1 and an increase in myostatin mRNA expression (Figure 19C and 19D). The mice treated with apocynin did not experience these cigarette smoke-induced changes in IGF-1 and myostatin mRNA expression. Specifically, with regards to atrogin-1 mRNA expression, there was a fold-change of 1.1, 0.7, and 0.7 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively compared to the sham + vehicle groups (Figure 19A). For MuRF-1, there was a fold-change of 1.4, 1.1, and 1.2 in
the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 19B). For IGF-1b, there was a fold-change of 1.2, 0.5, and 1.0 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 19C). For Myostatin, there was a fold-change of 0.9, 1.4, and 1.1 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 19D). This indicates that in this model, the skeletal muscle wasting observed was likely a result of a reduction in protein synthesis rather than an increase in protein degradation as demonstrated by the changes seen in expression of IGF-1 and Myostatin, which are implicated in muscle growth homeostasis pathways. Due to the pooling of samples, statistical analysis could not be performed for this data.
4.2.6. Assessment of mRNA expression in heart tissue

Due to the significant reduction in heart mass, we measured the expression of 4 genes known to contribute to various cardiovascular disorders, natriuretic peptide A (NPPA), myosin-1 (Myh1), tumour necrosis factor alpha (TNFα) and interleukin 1 alpha (IL-1α). There were no significant changes in expression in any of the genes, however there was a trend for IL-1α to
increase with smoke exposure. Specifically, with regards to NPPA mRNA expression, compared to the sham + vehicle groups, there was a fold-change of 1.10, 1.07, and 0.97 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 20A). For Myh-1, there was a fold-change of 1.40, 1.08, and 1.62 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 20B). For TNF-α, there was a fold-change of 1.17, 1.44, and 1.29 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 20C). For IL-1α, there was a fold-change of 1.16, 1.75, and 1.93 in the sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 20D). As differences observed did not reach significance in any of the data sets, it can be assumed that 8-weeks was likely not a long enough time for significant cardiovascular modifications to occur. This indicates that the reduction in heart mass observed in Table 1 may have just been a result of overall body mass reduction in the animals. Further analysis using more sensitive techniques may be required to observed and examine any cardiovascular modifications occurring in this model. Due to this, I sought to establish a mouse model for cardiac dysfunction following cigarette smoke exposure (refer to Chapter 5).
Figure 20. The effect of apocynin (5mg kg\(^{-1}\)) administration on heart tissue mRNA expression of NPPA, Myh1, TNF-\(\alpha\), and IL-1\(\alpha\) in mice after smoke exposure.

Data are expressed as mean ± SEM for n=8 mice per treatment group.

Due to insufficient sample volume and time constraints, ELISAs to measure systemic inflammation and systemic oxidative stress in this model could not be performed in Study A and muscle samples that were collected for histological examination could not be assessed. The collected tissues were preserved for future histological assessment. Lastly, functional testing of skeletal muscle in the mice was not available at this time. Following this
preliminary apocynin study, we replicated the same model with an increased sample size of n=12 to increase the statistical power of the study and increase the number of assays performed. Due to its efficacy in treating lung inflammation and protecting against skeletal muscle wasting, we hypothesised that the results observed with apocynin treatment would be reproduced.

4.3. Apocynin Study B

4.3.1. Assessment of BALF inflammation

In Study B, cigarette smoke exposure (6 cigarettes/day, 5 days/week for 8 weeks) significantly increased the total, macrophage and neutrophil number in BALF. However, in contrast to the first apocynin study (Figure 16), apocynin did not reduce cigarette smoke-induced increases in BALF total cells and macrophages (Figure 21A and 21B). However, smoke-exposed mice treated with apocynin had a significant reduction in neutrophils compared with vehicle treated smoke-exposed mice (Figure 21C) which is consistent with that observed in the first apocynin study (Figure 16C). Following the 56-day smoke exposure period, the number of total cells in the BALF was 1.61x10^5 ± 1.41x10^4, 1.74x10^5 ± 3.76x10^4, 5.37x10^5 ± 5.91x10^5, and 5.49x10^5 ± 4.76x10^4 cells/mL for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin respectively (Figure 21A). The number of macrophage cells was 1.58x10^5 ± 1.38x10^5, 1.79x10^5 ± 4.14x10^4, 4.42x10^5 ± 5.62x10^4, and 5.06x10^5 ± 5.10x10^4 cells/mL (Figure 21B), whilst the number of neutrophils was 2.57x10^3 ± 613, 1.96x10^3 ± 638, 9.38x10^3 ± 1.81x10^3, and 4.13x10^4 ± 1.13x10^4 cells/mL for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin respectively (Figure 21C).

Surprisingly the number of neutrophils across all groups was higher than those observed in the first apocynin study (A). There was approximately a 3-fold difference in the sham groups and a 2-fold difference in the smoke groups. This may be due to differences in air quality at the different animal facilities these studies were conducted in (Study A performed at The
University of Melbourne whereas Study B was performed at RMIT University given that my supervisor Prof Ross Vlahos moved to RMIT University during my PhD candidature). Lung PCR was not performed again in this repeat study due to time constraints.

**Figure 21.** The effect of apocynin (5 mg kg\(^{-1}\)) administration on BALF cellularity in mice after smoke exposure.

Balf cellularity is shown as (A) the total number of cells, (B) macrophages, (C) neutrophils. Data are expressed as mean ± SEM for n=12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.

### 4.3.2. Impact of smoke exposure on total body weight and food intake

In Study B, mouse body weight was measured each day and food consumption was measured Monday, Wednesday and Friday, with both measurements conducted at approximately the
same time per day. Smoke-exposed mice, regardless of treatment, experienced reduced growth compared to their sham counterparts. As seen in Figure 22A, which shows the average weekly weight of the animals, smoke-exposed mice treated with apocynin experienced a slightly improved rate of growth over the 56-day period compared to the vehicle treated group. However, unlike in study A when examining the final percentage change shown in Figure 22B, we see that there was no significant improvement in total body weight in the apocynin treated group. On day 56, the final increase in total bodyweight percentage was 16.1±0.8%, 14.4±1.1%, 1.97±1.7%, and 3.47±1.1% for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 22B).

Both smoke-exposed groups experienced a significant reduction in food intake compared to their sham counterparts over the 8-week period (Figure 22C). In addition, apocynin did not impact food intake. Food intake was 2.84±0.19, 2.83±0.19, 2.33±0.17, and 2.25±0.15 g/mouse/day for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively.
Figure 22. The effect of apocynin (5 mg kg\textsuperscript{-1}) administration on total body weight and food consumption in mice after smoke exposure.

(A) Body weight was recorded daily for each group over the 56-day period. (B) Final percentage weight change on day 56. (C) Food intake was calculated by measuring the amount of food remaining in the cages and dividing the difference between days by the number of mice in the cage. Data are expressed as mean ± SEM for n=12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle

4.3.3. Impact of smoke exposure on organ and skeletal muscle weights

Lungs

In this second study, the focus of the study was narrowed and we only collected the lungs, hind limb skeletal muscles, and WAT (Table 7). Lung weight was 282 ± 5.3 mg, 281 ± 6.6 mg, 305 ± 7.9 mg, and 300 ± 6.3 mg for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively. Smoke-exposure caused a significant
increase in lung weight, which was not reduced by treatment with apocynin. Compared to the sham + vehicle control, the smoke + vehicle, mean lung mass increased by 8.0% and the smoke + apocynin group by 6.3%. These values are considerably smaller than values acquired from the first study, which may be a reflection of the reduced inflammation across all groups in study 2.

**Skeletal Muscle**

In the second study, greater levels of wasting was observed as smoke exposure significantly decreased the weights of all 4 hind limb skeletal muscles and in both TWAT and RWAT (Table 6). Respectively for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups, muscle weights are as follows: Tibialis anterior: 60.5 ± 0.9 mg, 60.3 ± 1.3 mg, 54.0 ± 1.0 mg*, and 56.6 ± 1.6 mg. Soleus: 10.3 ± 0.3 mg, 10.2 ± 0.3 mg, 8.9 ± 0.3mg*, and 9.9 ± 0.4 mg. Plantaris: 19.1 ± 0.3 mg, 19.4 ± 0.5 mg, 16.1 ± 0.4 mg*, and 16.9 ± 0.5 mg*. Gastrocnemius: 149 ± 2.1 mg, 148 ± 2.2 mg, 131 ± 2.4 mg*, and 137 ± 2.2 mg*. Smoke-exposure significantly reduced the mean weight of the tibialis anterior, soleus, plantaris and gastrocnemius by 10.8%, 14.6%, 15.7%, and 12.2% respectively. Significant muscle wasting was not observed in the tibialis anterior and soleus muscles of the mice treated with apocynin, however, significant wasting was still observed in the plantaris and gastrocnemius. The tibias of the animals were collected and the length measured. The lengths of the TA and gastrocnemius were normalised to the tibia length, however this did not affect the statistical significance of the muscle wasting observed as a result of smoke-exposure. This indicates that the wasting observed was not proportional to the observed reduction in total body weight of the smoke-exposed mice.
White adipose tissue

With regards to adipose tissue mass, testicular WAT weights were 639 ± 37 mg, 529 ± 35 mg, 444 ± 57 mg*, and 381 ± 29 mg* and for retroperitoneal WAT, weights were 99.4 ± 5.3 mg, 74.5 ± 7.2 mg, 64.3 ± 9.3 mg*, and 54.6 ±4.4 mg* respectively for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups. Smoke-exposure significantly reduced the weight of the testicular WAT and retroperitoneal WAT by 30.6% and 35.4% respectively. Unlike the first study, treatment with apocynin did not protect mice from this smoke-induced reduction WAT.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SV</th>
<th>SA</th>
<th>CSV</th>
<th>CSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>27.3 ± 0.39</td>
<td>26.8 ± 0.48</td>
<td>23.6 ± 0.66 *</td>
<td>23.7 ± 0.27 *</td>
</tr>
<tr>
<td>Tibialis anterior (mg)</td>
<td>60.5 ± 0.9</td>
<td>60.3 ± 1.3</td>
<td>54.0 ± 1.0 *</td>
<td>56.6 ± 1.6</td>
</tr>
<tr>
<td>Soleus (mg)</td>
<td>10.4 ± 0.3</td>
<td>10.2 ± 0.3</td>
<td>8.9 ± 0.3 *</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>Gastrocnemius (mg)</td>
<td>149 ± 2.1</td>
<td>148 ± 2.2</td>
<td>131 ± 2.4 *</td>
<td>137 ± 2.2 *</td>
</tr>
<tr>
<td>Plantaris (mg)</td>
<td>19.1 ± 0.3</td>
<td>19.4 ± 0.5</td>
<td>16.1 ± 0.4 *</td>
<td>16.9 ± 0.5 *</td>
</tr>
<tr>
<td>Testicular WAT (mg)</td>
<td>639 ± 37</td>
<td>529 ± 35</td>
<td>444 ± 57 *</td>
<td>381 ± 29 *</td>
</tr>
<tr>
<td>Retroperitoneal WAT (mg)</td>
<td>99.4 ± 5.3</td>
<td>74.5 ± 7.2</td>
<td>64.3 ± 9.3 *</td>
<td>54.6 ± 4.4 *</td>
</tr>
<tr>
<td>Lung (mg)</td>
<td>282 ± 5.3</td>
<td>281 ± 6.6</td>
<td>305 ± 7.9 *</td>
<td>300 ± 6.2 *</td>
</tr>
</tbody>
</table>

Table 7. The effect of apocynin (5 mg kg⁻¹) administration on lung, heart, skeletal muscle, and WAT weights in mice after smoke exposure.

Data are expressed as mean ± SEM for n=12 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle
4.3.4. Assessment of tibialis anterior muscle function in situ

In the second apocynin study, the maximum force-frequency, rate of contraction, force generated at 120Hz, and the specific force at 120Hz were measured in situ using the 1300A: 3-in-1 Whole Animal System – Mouse force transducer apparatus as described in the Methods section (section 4.1.5) of this Chapter and the methods section in the General Methods Chapter (section 2.8.1).

**Force Frequency**

The frequency-dependent gain in contractility is an intrinsic property of both skeletal and cardiac muscle in mammals. As frequency increases, maximum force generated also increases until it reaches the peak threshold of possible force generation. In healthy mice, this peak occurs at approximately 120 Hz. As seen in Chapter 3 (Figure 9A), the frequency-dependent gain in contractile force was observed in the mice, with the peak occurring at 120 Hz for the sham + vehicle control group (Figure 23A). Following smoke exposure, the force-frequency relationship is altered, trending towards lower force generation across all frequencies. Additionally, the peak threshold is shifted in the smoke + vehicle animals, now occurring at 150Hz. This indicates that the function of the muscle may be impaired, which could be due to impaired mitochondrial function or the result of a shift in fibre type from type I (slow twitch) muscle fibres to type II (fast twitch) muscles known to occur in COPD [114]. We did not observed these trends in the mice treated with apocynin indicating that apocynin may have a protective effect against the contractile dysfunction in this model.

**Rate of Contraction**

As with the force-frequency relationship, there is also an intrinsic frequency-dependent gain in the rate of contraction of cardiac and skeletal muscle in mammals. As frequency increases,
the rate of contraction increases. In accordance with Figure 23A, the frequency dependent gain in the maximum rate of contraction was observed in all groups. Compared to vehicle treated groups, there was an observed trend showing that smoke exposure caused a reduction in maximum rate of contraction at all frequencies. Treatment with apocynin improved the maximum rate of contraction across all frequencies when compared to the smoke + vehicle group (Figure 23C). Specifically, at the initial and peak force-frequencies, there was a 14.5% improvement at 10 Hz, and a 13.8% improvement at 120 Hz with apocynin treatment. Once again, this data suggests that apocynin had a protective effect against contractile dysfunction in this model.

Max Force at 120Hz, Specific Force at 120Hz, and Absolute Max Force

Peak force generation occurred at 120Hz for all groups excluding the smoke + vehicle group. The maximum force at 120Hz did not significantly differ between the 4 groups, regardless of exposure or treatment (Figure 23B). Specifically, maximum force at 120Hz was 692 ± 74.7 mN, 632 ± 58.2 mN, 508 ± 74.1 mN, and 643 ± 31.7mN for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively. The smoke + vehicle group appears to have reduced force generation at 120 Hz, which corresponds to the observed shift in max force from 120 Hz to 150 Hz for this treatment group as seen in Figure 23A. The maximum force at 120 Hz was normalised using the $L_o$, which provided the specific force at 120 Hz.

Specific force was 161 ± 20.7 mN, 154 ± 12.5 mN, 141.2 ± 19.2 mN, and 160 ± 11.7 mN respectively (Figure 23D). As with the maximum force, the specific force did not significantly differ between the 4 groups, regardless of exposure or treatment. However, the same trend of reduced maximum force was observed in the smoke + vehicle group which also had reduced specific force generation at 120 Hz. To account for the discrepancy in the force-
frequency relationship of the smoke + vehicle group with the other groups, we examined the absolute maximum force generated regardless of frequency in the sham + vehicle and smoke + vehicle groups (Figure 23E).

Absolute maximum force was 669 ± 75.0 mN for the sham + vehicle group and 571 ± 79.8 mN for the smoke + vehicle group. Like the previous data, there is also an observed trend for reduced absolute maximum contractile force in the smoke-exposed mice.

Although not significant, this data shows a clear trend for impaired maximum contractile function because of smoke exposure that approaches significance. An increased sample size (n=20+) could offset the variance in contractile function observed.
Figure 23. The effect of apocynin (5mg kg⁻¹) administration on tibialis anterior contractile function in mice after smoke exposure.

(A) Maximum force-frequency relationship, (B) maximum rate of contraction, (C) maximum force generated at 120Hz, (D) specific force generated at 120Hz, and (E) maximum absolute force. Data are expressed as mean ± SEM for n=10-12 mice per treatment group.
4.3.5. Assessment of mRNA expression in skeletal muscle tissue

Tibialis anterior

In this study, the tibialis anterior and soleus muscles were protected against cigarette smoke-induced muscle wasting. Unlike study A the tibialis anterior tissues of this study were not pooled so statistical analysis could be performed. Additionally, due to difficulties in acquiring a sufficient RNA yield from soleus skeletal muscle tissues, the expression of mRNA in the soleus was not analysed.

The mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 was measured in both the tibialis anterior and gastrocnemius skeletal muscles. Refer to Table 8 for specific mRNA expression fold-change. In contrast to Study A, there were no alterations to the mRNA expression of any gene measured – specifically there were no alterations in IGF-1b or myostatin mRNA expression. As apocynin had a beneficial effect and acts via the inhibition of NOX-2 formation, the mechanism behind these beneficial effects is unclear as there is no clear driver of the observed wasting based on these results. It is possible that NOX-2 activity is increased, inflammatory cell infiltration into the muscle may be occurring, or ROS is being generated by another source (e.g. mitochondria) as a result of smoke exposure. Flow cytometry or immunohistochemistry should be performed in future studies to determine if there is inflammatory cell infiltration in the skeletal muscle of the animals as a result of this smoke-exposure model.
Table 8. The effect of apocynin (5 mg kg\(^{-1}\)) administration on tibialis anterior tissue mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 in mice after cigarette smoke exposure.

Data are expressed as mean ± SEM for n=3-11 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham Vehicle</th>
<th>Apocynin</th>
<th>Cigarette Smoke Vehicle</th>
<th>Apocynin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>1.00 ± 0.21</td>
<td>1.10 ± 0.23</td>
<td>1.03 ± 0.15</td>
<td>1.02 ± 0.15</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>1.00 ± 0.15</td>
<td>0.99 ± 0.20</td>
<td>0.91 ± 0.20</td>
<td>0.95 ± 0.22</td>
</tr>
<tr>
<td>IGF-1eA</td>
<td>1.00 ± 0.14</td>
<td>1.09 ± 0.30</td>
<td>0.96 ± 0.30</td>
<td>0.97 ± 0.20</td>
</tr>
<tr>
<td>IGF-1eB</td>
<td>1.00 ± 0.11</td>
<td>1.12 ± 0.14</td>
<td>1.14 ± 0.14</td>
<td>0.95 ± 0.11</td>
</tr>
<tr>
<td>Myostatin</td>
<td>1.00 ± 0.12</td>
<td>1.01 ± 0.15</td>
<td>0.96 ± 0.15</td>
<td>0.97 ± 0.09</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00 ± 0.16</td>
<td>0.75 ± 0.09</td>
<td>0.64 ± 0.09</td>
<td>0.99 ± 0.22</td>
</tr>
<tr>
<td>NOX-2</td>
<td>1.00 ± 0.16</td>
<td>0.88 ± 0.12</td>
<td>0.86 ± 0.08</td>
<td>0.87 ± 0.11</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>1.00 ± 0.11</td>
<td>1.24 ± 0.18</td>
<td>1.34 ± 0.26</td>
<td>1.03 ± 0.11</td>
</tr>
<tr>
<td>Nfe2l2</td>
<td>1.00 ± 0.14</td>
<td>1.10 ± 0.14</td>
<td>1.07 ± 0.11</td>
<td>1.09 ± 0.16</td>
</tr>
</tbody>
</table>

Gastrocnemius

In this study, there was significant wasting of the gastrocnemius due to cigarette smoke exposure. Although significant improvements in gastrocnemius mass were not observed following treatment with apocynin, significant wasting was still observed in these tissues which warranted analysis to help elucidate the mechanism by which this wasting occurred. Unlike study A, which only explored mRNA expression in the tibialis anterior, the mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 was also measured via qRT-PCR. Refer to Table 9 for specific mRNA expression fold-change. As with the tibialis anterior tissue, there were no significant alterations to the mRNA expression of any gene measured. As these observations were in concordance with the skeletal muscle mRNA expression data in Chapter 3, it is likely that the wasting observed in this smoke exposure model is primarily driven by direct muscle damage rather than alterations to the protein homeostasis pathway.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham Vehicle</th>
<th>Apocynin</th>
<th>Cigarette Smoke Vehicle</th>
<th>Apocynin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>1.00 ± 0.14</td>
<td>1.08 ± 0.18</td>
<td>0.91 ± 0.12</td>
<td>0.91 ± 0.12</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>1.00 ± 0.14</td>
<td>0.90 ± 0.12</td>
<td>0.87 ± 0.12</td>
<td>0.91 ± 0.11</td>
</tr>
<tr>
<td>IGF-1eA</td>
<td>1.00 ± 0.18</td>
<td>1.12 ± 0.15</td>
<td>0.84 ± 0.08</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td>IGF-1eB</td>
<td>1.00 ± 0.10</td>
<td>1.29 ± 0.16</td>
<td>1.08 ± 0.08</td>
<td>1.17 ± 0.16</td>
</tr>
<tr>
<td>Myostatin</td>
<td>1.00 ± 0.25</td>
<td>0.78 ± 0.11</td>
<td>0.86 ± 0.09</td>
<td>0.98 ± 0.21</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00 ± 0.13</td>
<td>0.94 ± 0.12</td>
<td>0.88 ± 0.13</td>
<td>1.09 ± 0.17</td>
</tr>
<tr>
<td>NOX-2</td>
<td>1.00 ± 0.21</td>
<td>1.05 ± 0.17</td>
<td>0.85 ± 0.11</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>1.00 ± 0.21</td>
<td>0.87 ± 0.11</td>
<td>0.85 ± 0.07</td>
<td>1.10 ± 0.24</td>
</tr>
<tr>
<td>Nfe2l2</td>
<td>1.00 ± 0.15</td>
<td>1.04 ± 0.15</td>
<td>0.90 ± 0.06</td>
<td>1.02 ± 0.13</td>
</tr>
</tbody>
</table>

Table 9. The effect of apocynin (5 mg kg\(^{-1}\)) administration on gastrocnemius tissue mRNA expression of atrogin-1, MuRF-1, IGF-1eA, IGF-1eB, myostatin, IL-6, NOX-2, PPARGC1A, and NFE2L2 in mice after cigarette smoke exposure.

Data are expressed as mean ± SEM for n=6-11 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.

4.3.6. Markers of systemic inflammation and oxidative stress

C-reactive protein

C-reactive protein was measured as a marker of systemic inflammation. In this study, CRP was 3.17x10\(^3\) ± 247 ng/mL, 3 75x10\(^3\) ± 169 ng/mL, 4.43x10\(^3\) ± 349 ng/mL*, and 5.68 ± 356 ng/mL*# for sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 24A). Smoke exposure caused significant increases in serum CRP levels, which was not improved by treatment with apocynin but rather worsened with treatment. Specifically, compared to the sham + vehicle group, there was an increase of 34.0% in the smoke + vehicle group and an even greater 90% increase in the smoke + apocynin group. This result was unexpected, and it is possible that the combination of cigarette smoke and inhibition of NOX-2 by apocynin had a negative effect on systemic
regulation of CRP. Additionally, to our knowledge, no other published studies have shown this phenomenon.

**TBARS (MDA)**

Malondialdehyde (MDA) was measured as a marker of systemic oxidative stress. In this study, smoke exposure caused a significant increase in MDA concentration in the serum of both treatment groups. MDA concentration was 49.0 ± 3.7 ng/mL, 43.7 ± 3.4 ng/mL, 59.6 ± 5.9 ng/mL, and 68.1 ± 8.7 ng/mL for the sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively (Figure 24B).

**8-isoprostane**

Isoprostanes are a family of eicosanoids produced by the random oxidation of tissue phospholipids by ROS. They are detected in plasma and urine under normal conditions and are elevated by oxidative stress. 8-Isoprostane has been proposed as a marker of antioxidant deficiency and oxidative stress. Additionally, elevated levels of 8-isoprostane have been found in heavy smokers.

Although the serum concentration of MDA was elevated with cigarette smoke exposure, the concentration of 8-isoprostane was unchanged, (Figure 24C) which was unexpected as both are markers of oxidative stress. This result may be due to the degradation of the serum samples due to the high number of freeze-thaw cycles undergone by the samples by the time the 8-isoprostane ELISA was performed.
Figure 24. The effect of apocynin (5 mg kg\(^{-1}\)) administration on markers of systemic inflammation and systemic oxidative stress in mice after cigarette smoke exposure.

(A) Levels of C-reactive protein (CRP), (B) malondialdehyde (MDA) and (C) 8-isoprostane in serum. Data are expressed as mean ± SEM for n=7-8 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle, #P<0.05 vs Smoke + Vehicle.

4.3.7. Assessment of oxidative stress in gastrocnemius tissue

As with study A, protein was extracted from gastrocnemius tissues and protein carbonylation was measured using an oxyblot chemiluminescence assay as a marker of oxidative stress. Gastrocnemius tissue was used as all the tibialis anterior tissue had been consumed for gene expression analysis. Protein carbonylation was then quantified and normalised to β-actin. Figure 25A shows a representative lane for each treatment group and it was apparent from...
this alone that cigarette smoke caused a drastic increase in protein carbonylation in the tissue sample. Cigarette smoke caused a significant increase in protein carbonylation compared to the sham vehicle group. Specifically, protein carbonylation densitometry of the gastrocnemius tissue was measured to be $7.80 \times 10^6 \pm 6.3 \times 10^5$ Int, $7.67 \times 10^6 \pm 6.16 \times 10^5$ Int, $1.82 \times 10^7 \pm 2.1 \times 10^6$ Int*, and $1.25 \times 10^7 \pm 2.4 \times 10^6$ Int* for sham + vehicle, sham + apocynin, smoke + vehicle, and smoke + apocynin groups respectively after normalisation to β-actin (Figure 25B). Treatment with apocynin did not result in a significant reduction in smoke-induced protein carbonylation however it trended towards significance (P=0.09). Due to time constraints, the mouse skeletal muscle samples that were collected for histological examination were not examined.

![Figure 25. The effect of apocynin (5 mg kg⁻¹) administration on protein carbonylation in gastrocnemius tissue from mice after smoke exposure.](image)

(A) Representative oxyblot showing differences in smearing between treatment groups. (B) Densitometry analysis of oxyblots normalised to β-actin. Data are expressed as mean ± SEM for n=3-4 mice per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs Sham + Vehicle
4.4. **C2C12 *In Vitro* pilot studies**

The following pilot studies were conducted to explore the mechanisms that may be driving the changes in skeletal muscle mass and function seen *in vivo* because of increase oxidative stress. The C2C12 cell line is a sub-clone (produced by H. Blau, et al [299]) of the mouse myoblast cell line established by D. Yaffe and O. Saxel [300]. This cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. Initial pilot studies to highlight the direct impact of cigarette smoke and, more specifically, oxidative stress were conducted using this cell line.

4.4.1. **Impact of H$_2$O$_2$ on C2C12 skeletal muscle cell viability**

As seen in Figure 12, exposure to supra-physiological concentrations of H$_2$O$_2$ caused a significant reduction in C2C12 cell viability at both 1 and 3-hour time points. Exposure to concentrations ranging from 30 μM to 100 μM resulted in an approximate 75% reduction in cell viability. Specifically, at 1-hour H$_2$O$_2$ stimulation, absorbance reading was 1.18 ± 0.4 AU, 1.17 ± 0.4 AU, 0.630 ± 0.6 AU, 0.570 ± 0.3 AU, and 0.300 ± 0.2 AU for 0, 10, 30, 100, and 300 μM concentrations of H$_2$O$_2$ respectively (Figure 26). Concentrations of H$_2$O$_2$ greater than 100 μM resulted in complete cell death. Based on this data, there was a clear shift in cell viability that occurred between 10 μM and 30 μM; thus, future studies used 10-30μM concentrations of H$_2$O$_2$ for C2C12 stimulation.
Data are expressed as mean ± SEM for n=3 samples per treatment group. Two-way ANOVA with Bonferroni post-hoc test was performed to assess statistical significance. *P<0.05 vs control

**Figure 26. The effect of increasing H$_2$O$_2$ concentrations on C2C12 cell viability at 1 and 3 hours post treatment.**

4.4.2. **Impact of H$_2$O$_2$ on mRNA expression in C2C12 cells**

C2C12 cells were lysed, pooled and RNA was extracted. The expression of TNF-$\alpha$ and IL-6 was measured via qRT-PCR. An approximate 3-fold increase in mRNA expression of both genes was observed following stimulation with 10 μM H$_2$O$_2$ (Figure 27). Due to the pooling of samples, statistical analysis could not be performed for this data, however, these experimental parameters have since been explored by Mastronardo et al. (unpublished) who showed the same observed increase in mRNA expression of both TNF-$\alpha$ and IL-6 with statistical significance. This indicates that small increases in oxidative stress are sufficient to induce acute changes in skeletal muscle mRNA expression of inflammatory cytokines.
4.4.3. Impact of H$_2$O$_2$ on mitochondrial respiration in C2C12 cells

As mitochondria are an important source of intracellular ROS, an extracellular flux mitochondrial stress test was performed to determine the effect of supraphysiological concentrations of H$_2$O$_2$ on mitochondrial function. Impaired mitochondrial function can result in impaired muscle function and increased intracellular oxidant generation. Specifically, basal respiration, ATP-linked respiration, electron transport chain proton leakage, maximal respiration, and mitochondrial reserve capacity were measured in C2C12 mouse skeletal muscle cells (Figure 28). To account for any cell death following H$_2$O$_2$ exposure, data was normalised to the total protein content of cells in the assay plate. At 10 μM, basal respiration was increased by 79%, proton leakage was increased by 449%, maximal respiration was decreased by 25%, and reserve capacity was decreased by 83%. At 30 μM, ATP-linked respiration was decreased by 57%, proton leakage was increased by 275%, maximal respiration was decreased by 72% (to basal levels), and reserve capacity was completely
eliminated. This data indicates that oxidative stress indiscriminately impaired mitochondrial respiration along all processes of the electron transport chain. Although the observed trends show impaired mitochondrial function, the small sample size is inadequate to provide well-defined conclusions and future studies should use a greater sample size.

The mitochondrial impairment reported above may be one mechanism contributing to reduced contractile function observed in mice following chronic cigarette exposure (Figure 9) however further exploration is required. Future studies should explore mitochondrial function in primary skeletal muscle tissue extracted from mice following a 56-day cigarette smoke exposure protocol.

Figure 28. The effect of 10 and 30 μM H₂O₂ on C2C12 respiration after 1 hour of exposure.

Data are expressed as mean ± SEM for n=2 samples per treatment group. Due to the small sample size, statistical analyses could not be performed on this data.
4.5. Discussion

This is the first study to show the successful inhibition of cigarette smoke-induced lung inflammation and skeletal muscle wasting due to inhibition of NOX-2 via treatment with apocynin, in an established animal model of COPD. This data supports the potential benefits of targeting oxidant dependent mechanisms for dual therapy to treat both pulmonary and systemic manifestations of COPD.

Our findings showed that mice exposed to sub-chronic cigarette smoke have enhanced BALF inflammation whereas treatment with apocynin attenuated this increase in inflammation. As seen in previous studies [207], smoke exposed mice, regardless of treatment, had a significant increase in total cells, macrophages and neutrophils in the BALF (Figure 16 and 21). In both Study A and B, smoke exposed mice treated with apocynin experienced a significant reduction in neutrophil numbers of approximately 50-55% however, significant reductions in total cells and macrophages were only observed in Study A (Figure 16B). This reduction in neutrophils was similar in magnitude to that seen in acute smoke exposure studies and that seen in Chapter 3 and is consistent with the 50% decrease in neutrophil counts in BALF observed in animals treated with 5mg/kg of apocynin per day for 7 days following cigarette smoke exposure and inoculation with influenza A virus [229]. It should be noted that a slight but significant reduction in macrophages and overall total cell counts in BALF was also observed in an acute smoke exposure model as a result of apocynin treatment [229] and this was not observed in our findings. The similarity between the BALF cell counts in the vehicle treated and apocynin treated sham-exposed mice indicates that inhibition of NOX-2 has no effect on BALF cellularity in healthy mice. The significant increase in lung mass observed in all smoke exposure groups can be attributed to increased inflammation and pulmonary oedema. As per chapter 3, airspace enlargement was not determined in this model.
Consistent with the observations in Chapter 3, smoke exposure caused an increase in expression of several pro-inflammatory cytokines and chemokines in Study A (IL-17A, MIP-2α, MCP-1, and CXCL-10) and treatment with apocynin reversed some of these smoke induced alterations (Figure 17 A-D). It should be noted that lung mRNA expression was not assessed in Study B due to time limitations. MCP-1 and CXCL-10 are important monocyte recruitment factors responsible for macrophage accumulation [239, 240, 301]. The pooled data showed an increase in MCP-1 and CXCL-10 gene expression in lung tissue of smoke-exposed vehicle treated mice and this increase likely explains the very large infiltration of macrophages in the lungs. Additionally, treatment with apocynin reduced expression of both genes and may explain the reduction in macrophages in Study A.

MIP-2α is a chemokine, secreted by macrophages, which acts as a polymorphonuclear leukocyte chemoattractant and IL-17A is a cytokine involved in activating neutrophil-mobilizing factors such as IL-8 and GM-CSF and hence sustaining the accumulation and activity of neutrophils locally in the lungs [243, 302, 303]. The elevation of IL-17A and MIP-2α may explain the increase in BALF neutrophil numbers; however, the apocynin treated smoke-exposed animals still exhibited elevated expression of these genes. This indicates that other factors are driving the significant reduction in BALF neutrophils seen in the apocynin treated smoke-exposed mice. Some potential mechanisms driving the influx of neutrophils and the subsequent reduction in the apocynin treated mice could be other neutrophil chemoattractants that were not measured, and/or the efferocytosis of neutrophils by alveolar macrophages. It is known that macrophage efferocytosis is reduced in COPD and it is possible that apocynin restored alveolar macrophage phagocytic ability back to baseline, promoting clearance of neutrophils [304, 305]. Further experiments need to be conducted to determine the exact mechanism. Regardless of mechanism of action, the effect of apocynin on BALF cellularity and inflammatory mediator expression indicates that
inhibiting NOX-2 may be of great therapeutic benefit for the treatment of pulmonary manifestations in COPD.

In addition to the pulmonary inflammation that is characteristic of the disease, COPD patients exhibit chronic systemic inflammation [246, 247]. Systemic inflammation is associated with an accelerated decline in lung function [248] and is also a driver of systemic oxidative stress, which in turn further perpetuates systemic inflammation [102]. This vicious cycle leads to systemic manifestations of disease in the form of comorbidities, such as peripheral skeletal muscle wasting [112].

Serum concentration of CRP, an acute phase protein that is known to have elevated levels in COPD patients, was measured as a marker of systemic inflammation in this model. As expected, serum CRP levels were elevated in response to smoke exposure, however, treatment with apocynin actually caused a further increase in CRP concentration rather than a decrease (Figure 24A). This is in contrast to the reduced BALF cellularity observed in the treatment animals, which showed an improvement in smoke-induced neutrophilic inflammation (Figure 16C and 21C). This suggests that although lung inflammation is hypothesized to spillover into systemic circulation in COPD [249], this phenomenon may not be a key driver of systemic inflammation in this model and, subsequently, lung inflammation is likely not a key driver of systemic manifestations of disease either. This indicates that the observed systemic inflammation is likely originating outside of the lungs and is consistent with the hypothesis that systemic inflammation in COPD instead originates from a parallel inflammatory response in other tissues, such as adipose tissue [250, 251]. In addition to the increase in serum CRP, there was also a significant increase in MDA concentration in serum following smoke exposure (Figure 24B). An increase in plasma MDA concentration is among the most common and consistent alterations experienced in COPD and several studies have shown that increases in MDA occur in parallel to increasing severity of disease [306-308]. To
our knowledge, this is the first time that the characteristic increase in circulating MDA observed in COPD patients has been induced in a cigarette smoke exposure animal model of COPD. Inhibition of NOX-2 with apocynin did not attenuate this increased systemic oxidative stress indicating that while sufficient for the treatment of pulmonary pathologies, the dose used may have been inadequate for the reduction of circulating oxidative stress. Alternatively, the systemic oxidative stress may not be NOX-mediated and further experiments will be required to determine the exact mechanism and origin.

Both acute and chronic smoke exposure is known to reduce total body mass in animal models [252, 253]. As expected, and as observed in Chapter 3, the smoke exposed animals experienced a significant reduction in total body weight compared to their sham exposed counterparts (Figure 18 and 22). In Study A, apocynin was able to significantly improve the body weights of animals however this observation was not replicated in Study B (Figure 18B). Additionally, smoke exposure caused a significant reduction in adipose tissue mass and apocynin was only able to attenuate this reduction in Study A (Table 6), which explains why there was no significant difference in total body weights of smoke-exposed animals in Study B (Table 7). These conflicting results between the two apocynin studies make it difficult to draw meaningful conclusions regarding the efficacy of apocynin on smoke induced total body weight reduction.

While the appetite suppressing effect of cigarette smoke is well established [254] and may possibly account for the reduction in body weight and skeletal muscle masses, it has been previously shown by using long-term pair-feeding that cigarette smoke exposure causes a greater reduction in body weight compared to equivalent food restriction alone (smoke exposure protocols of 1 and 3 months) [147, 207]. In this study, the smoke-exposed animals had a suppressed appetite that was unaffected by treatment with apocynin (Figure 18C and 22C). Additionally, apocynin did not impact the appetite of sham-exposed animals, indicating
that any alterations to the disease pathology and/or physiology of the animals cannot be attributed to increased food intake/caloric surplus in the treatment groups.

Although overall body mass was reduced due to smoke exposure, lung mass was significantly increased as expected (Table 6 and 7). Surprisingly, cigarette smoke caused a significant reduction in heart mass that was not improved by apocynin. This is in contrast to the hypertrophy of cardiac tissue widely observed in the hearts and vasculature of COPD patients that arises from cardiovascular manifestations of disease [255, 256]. As there were no significant alterations to mRNA expression of factors that may be driving alterations to cardiac mass (Figure 20), 8-weeks of smoke exposure is likely insufficient to induce significant cardiovascular modifications. This indicates that the significant reduction in heart mass may simply be driven by the overall body mass reduction in the animals. Further analysis using more sensitive techniques may be required to observe and examine modifications cardiovascular morphology and function that may be occurring in this model. The design and development of a suitable technique is described in Chapter 5.

Peripheral skeletal muscle wasting is highly prevalent in COPD and is associated with reduced quality of life, decline in lung function and increased mortality [112, 257]. Increased inflammation and oxidative stress both contribute to the onset and development of skeletal muscle wasting [212, 258]. In this study, smoke exposure caused a significant reduction in mass of the hind limb muscles, primarily in the tibialis anterior and the soleus. It should be noted that significant wasting was also observed in the gastrocnemius and plantaris muscles of smoke-exposed animals in Study B (Table 7). The observed wasting of the smaller gastrocnemius complex muscles (the soleus and plantaris) is consistent the reports of others [259-261]. This is contrast to our findings from Chapter 3 where smoke exposure did not induce wasting in the smaller gastrocnemius muscles (Table 2 and 3). This more severe response to smoke-exposure, as shown by the significant increases in systemic oxidative
stress, may be magnifying the skeletal muscle alterations in this study (Figure 24B). Treatment with apocynin prevented wasting in all hind limb muscles except the gastrocnemius (Table 6 and 7). This indicates that while NOX-2 inhibition did not improve systemic oxidative stress, it did improve local skeletal muscle alterations induced by smoke exposure. These findings highlight the possibility that systemic inflammation and systemic oxidative may only be involved in the onset of muscle wasting and they may not be involved in the ongoing progression of the comorbidity. This is consistent with the fact that COPD patients generally have relatively low concentrations of systemic inflammation and oxidative stress biomarkers even when these biomarkers are elevated in skeletal muscle [101, 286, 287]. While the link is well established, further studies are required to determine the exact contribution that systemic inflammation and systemic oxidative stress have in the pathology of comorbid skeletal muscle wasting.

Skeletal muscle dysfunction commonly accompanies skeletal muscle wasting in COPD [262]. Whole muscle contractility was used to assess TA muscle function in Study B. In contrast to the results observed in Chapter 3, there was a trend to altered skeletal muscle function in response to smoke exposure (Figure 23). Specifically, smoke exposure reduced TA contractility, endurance and force generation. This was consistent with the significant wasting observed in the TA muscle following smoke exposure. Additionally, treatment with apocynin was improved contractility and force generation, which was consistent with the improvements to TA mass. This echoes the observations seen in COPD patients where impaired skeletal muscle function is a reflection of skeletal muscle wasting severity [309, 310]. This demonstrates that by treating skeletal muscle wasting, we were also able to improve the associated skeletal muscle dysfunction via treatment with a single antioxidant compound.
Current literature has mostly focused on the catabolic mechanisms in skeletal muscle, specifically the ubiquitin-proteasome pathway. This pathway is one of the major atrophy-related pathways in skeletal muscle and is widely studied in cachexia, sarcopenia and comorbid skeletal muscle wasting [273]. Atrogin-1 and MuRF-1 are E3 ubiquitin ligases that are key regulators of ubiquitin-mediated protein degradation in skeletal muscle [274, 275]. Increased transcription of these ligases results in downstream increases in protein degradation and drives atrophy of the muscle if chronically elevated. This is evident in biopsies taken from the quadriceps of COPD patients [276, 277]. In this study, smoke exposure had no significant effects on the mRNA expression of the E3 ligases Atrogin-1 or MuRF-1 in the tibialis anterior and gastrocnemius muscles (Figure 19A, 19B, Table 8 and 9). When examining the alterations to expression of proteins involved in hypertrophy-related pathway, we were presented with inconsistent observations between Study A and Study B. In the pooled TA tissue from Study A, we saw a decrease in Insulin-like Growth Factor-1 (IGF-1) and an increase in myostatin expression (Figure 19C and 19D). As discussed in Chapter 1, IGF-1 initiates the protein synthesis pathway leading to increased muscle growth [311, 312]. On the other hand, myostatin acts as part of the atrophy-related process but instead of promoting protein degradation, it inhibits AKT in the IGF-1 pathway leading to reduced protein synthesis (Refer to Chapter 1, Figure 5) [313]. This indicates that smoke-exposure affected both sides and shifted the homeostatic balance towards protein degradation. Treatment with apocynin prevented these smoke-induced alterations, which is consistent with its ability to prevent wasting in the TA and soleus muscles in this study. This indicates that a reduction in muscle protein synthesis, rather than an increase in muscle protein degradation, may be the primary driver of the wasting observed in this model. These findings are in contrast to what was observed in Study B where smoke exposure had no effect on mRNA of other proteins involved in atrophy or hypertrophy related pathways, including myostatin and
IGF-1 (Table 8 and 9). This indicates that the reduction in muscle mass in the replicate study was not driven by the characteristic alterations to protein homeostasis signalling pathways observed in COPD patients. Additionally this data was not generated using pooled tissue unlike Study A, and is consistent with the findings from Chapter 3. As such, it is likely that the mRNA alterations observed in Study A are not representative of the underlying mechanisms of the skeletal muscle wasting induced by this model. While the alterations to the muscle homeostatic pathway gene expression were inconsistent, increased oxidative stress in the gastrocnemius following smoke exposure was observed in Study B. This will be addressed later in this thesis.

Similar to the observations reported in Chapter 3, there were no alterations to the mRNA expression of the inflammatory mediator IL-6 indicating that while there was a significant increase in pulmonary inflammation in the smoke exposed mice, the significant reduction in skeletal muscle mass was not being driven by a similar local inflammatory response in the muscles. Additionally, mitochondrial Nfe2l2 and PARGC1A expression was also unaltered indicating that the impaired muscle function in response to smoke-exposure is driven by reductions in muscle mass rather than altered mitochondrial function. Lastly, NOX-2 expression was unaffected following smoke-exposure. This indicates that skeletal muscle NOX-2 levels are not increased in response to cigarette smoke; however, this does not necessarily mean NOX-2 activity is unaltered. Superoxide generation in skeletal muscle may have increased, however, further assessments are required to determine this. Treatment with apocynin did not affect NOX-2 expression and this was expected as apocynin acts by inhibiting the assembly of NOX subunits rather than altering mRNA expression or transcription of the proteins [314].
While there is no strong relationship between muscle oxidative stress and local inflammation in patients with COPD [101, 285], 8 weeks of smoke exposure induced a significant increase in skeletal muscle oxidative stress in this model as measured by protein carbonylation in the gastrocnemius (Figure 25). Similar to the observations in Chapter 3, these oxidative alterations were not accompanied by a marked increase in local inflammation (as measured by IL-6 mRNA expression), which echoes what is seen in COPD. There was a significant increase in protein carbonylation following smoke exposure, which was reduced by treatment with apocynin (Figure 25B). This implies that unlike ebselen, treatment with apocynin was able to improve pulmonary inflammation and also prevent oxidative damage in the skeletal muscles of the animals. As with Chapter 3, this data indicates that oxidative stress a key driver of muscle wasting in this model and that targeting oxidative stress was able to significantly improve both pulmonary and skeletal muscle manifestations of disease.

In addition to the in vivo model of disease, we sought to establish an in vitro model of oxidative stress-induced skeletal muscle wasting that could be used to identify the specific mechanisms driving the alterations to skeletal muscle in COPD. This pilot study was conducted to determine the appropriate concentrations of H$_2$O$_2$ required to alter skeletal muscle morphology, create a pro-inflammatory environment, and induce impaired function in the C2C12 mouse skeletal muscle cell line. Published studies exploring the effects of oxidative stress on this cell line have generally used high concentrations of H$_2$O$_2$ with acute exposure times [315-317]. As we required alterations to morphology, mRNA expression and mitochondrial function, acute exposure was likely sufficient to only alter mRNA expression and mitochondrial function but not cell morphology. As such, the effect of various exposure times and stimuli concentrations was explored to determine the appropriate conditions.
Concentrations of H$_2$O$_2$ greater than 100µM resulted in complete cell death regardless of length of exposure (Figure 26). Additionally, there was a clear shift in cell viability that occurred between 10µM and 30µM and at both 1 hour and 3 hours of exposure. Thus, a concentration range of 10-30µM and 1 hour of exposure was determined to be sufficient to explore the impact of H$_2$O$_2$ on mRNA expression and mitochondrial function without significant impact to cell viability. Preliminary microscopic analysis showed no distinct signs of cell degradation or cell debris indicating these conditions were suitable for assessing alterations in morphology. In pooled samples, these conditions were able to cause increased expression of both IL-6 and TNF-α, pro-inflammatory cytokines that are known to be increased locally and systemically in COPD (Figure 27). This indicates that small increases in oxidative stress are sufficient to induce acute changes in skeletal muscle mRNA expression of inflammatory cytokines. Additionally, H$_2$O$_2$ exposure indiscriminately impaired mitochondrial respiration along all processes of the electron transport chain (Figure 28). These results validate the use of this in vitro model for assessing the specific effects of oxidative stress in skeletal muscle, the mechanisms involved in protein synthesis and degradation, and the impact of antioxidant treatment on these mechanisms. Due to time limitations, we were unable to conduct further experiments for this thesis using this model; however, this model has been successfully used in other studies conducted by our group (Mastronardo et al, unpublished).

In summary, we have shown that inhibition of NOX-2, via treatment with apocynin, is an effective therapy for both pulmonary and skeletal muscle manifestations of smoke exposure in this model of COPD. Apocynin successfully reduced smoke-induced increases lung inflammation, lung inflammatory cytokine and chemokine mRNA expression, and lung mass. Apocynin also reduced smoke-induced skeletal muscle and WAT wasting, improved muscle contractility and function, and reduced oxidative damage to muscle tissue. This pre-
clinical study presents a strong case for the viability of treating both COPD and its associated comorbidities with a single antioxidant compound.
Chapter 5: Evaluation of right heart function in a rat model using modified echocardiographic views
5. Introduction

This chapter was published in PLOS One (2017) with the same title, “Evaluation of right heart function in a rat model using modified echocardiographic views”. The full publication can be found in the appendices. The manuscript was slightly modified to match the format of this thesis. Following the observed cardiovascular alterations in chapters 3 and 4, I sought to explore cardiovascular manifestations of COPD in greater detail. Upon review, we determined that current rodent imaging techniques were not suitable to provide detailed observation and analysis of cardiac morphology and function when compared to techniques performed in human patients. The aim of this study was to adapt the Scherrer-Crosbie (SC) short axis (SAX) view to transthoracic examination in the rat, and compare right ventricle (RV) assessment at the mid-ventricular SAX level, with the aortic SAX view of the right ventricular outflow tract (RVOT). Additionally, the suitability of the modified SC view for 2D-strain analysis was investigated.

Animal models provide valuable insight into the pathophysiology of chronic lung disease, and enable assessment of potential therapeutics for conditions such as chronic obstructive pulmonary disease (COPD) [318, 319]. Animal exposure to chronic hypoxia, monocrotaline injury or pulmonary trunk banding, have contributed greatly to understanding the relationship between lung injury and progressive dysfunction of the right ventricle (RV) [320]. In many studies echocardiography plays a major role in assessing RV function and the efficacy of therapeutic intervention. Transthoracic views of the RV include the short-axis (SAX) mid-ventricular and aortic RV outflow views (RVOT), and the long-axis (LAX) apical 4-chamber view. Recent advances in ultrasound technology, including 2D speckle tracking or strain, have seen these modalities now being applied to clinical practice[321]. However the
application of 2D-Strain in small animals is emerging, and has been recently reported for left ventricular assessment [322]. A limitation of transthoracic imaging in small animals is the effect of anatomical artifact from the thoracic cage or liver on image quality. Suboptimal imaging limits RV assessment with standard views, and can prevent the application of advanced analysis including 2D-strain imaging.

In an alternative approach, Scherrer-Crosbie et al developed a transoesophageal technique in mice, where the right ventricular wall and chamber were evaluated in the mid-ventricular SAX view [210]. Significantly, echocardiographic findings were validated with ultrasound flow-measurement and magnetic resonance imaging (MRI). This modified RV approach is relevant to current clinical guidelines, which advocate the use of a RV-focused view over the standard four-chamber view. Optimal RV assessment occurs when imaging allows visualization of most or entire RV free wall [323].

In this study we aimed to adapt the Scherrer-Crosbie SAX (SC) view to transthoracic examination in the rat, and compare RV assessment at the mid-ventricular SAX level, with the aortic SAX view of the right ventricular outflow tract (RVOT). The RVOT view is a valuable window for assessing Doppler-derived RV ejection. In addition I plan to investigate whether the modified SC view is suitable for 2D-strain analysis.

5.1. Methods

Studies were conducted in accordance with National Health and Medical Research Council of Australia guidelines, and approved by the University of Melbourne Animal Ethics Committee (Ethics No. 1212675). All animals were acquired from the Animal Resource Center, Perth, Australia.
5.1.1. Echocardiographic Analysis of Ventricular Size and Function

Male WKY rats (300-350g) were anesthetized with 2.5% isoflurane, spontaneously ventilated and placed on a heated pad in the semi-left lateral position with upright tilt, suitable for echocardiographic examination. Pulse rate, oxygen saturation and temperature were continuously monitored. After shaving, a sequential examination of the left and right ventricles was performed using a Vivid E9 with i13-L (6-14MHz) linear array transducer (GE Vingmed Ultrasound AS, Horten, Norway). Each study imaged the PLAX and mid-papillary SAX views of the LV, and the modified Scherrer-Crosby and aortic RVOT/pulmonary artery SAX view of the RV (Figure 29). LV morphology was assessed for interventricular septal (IVS) wall and posterior wall (PW) thickness, LV end-diastolic chamber dimension (LVEDD), LV end-systolic chamber dimension (LVESD), fractional shortening (FS), LV end-diastolic chamber area (LVEDA), LV end-systolic chamber area (LVESA) and fractional area change (FAC). RV chamber width was assessed at mid-chamber in the SC view (R1) and RVOT width measured in the aortic SAX view (R2). In this view the main pulmonary artery diameter (PAφ) was also measured. RV wall area was measured in both SC and RVOT SAX views calculated from the difference between traced epicardial and endocardial areas [210]. Right ventricular ejection was measured in the aortic SAX view at the proximal MPA (PA diameter) using 2D-Doppler and the waveform analyzed for pulmonary artery velocity-time integral (VTI) and time to peak ejection (PAAT), Pulmonary artery systolic pressure (PASP) was not assessed (TR jet not examined).
Figure 29. Echocardiographic views used to assess cardiac function

a. Parasternal long-axis (PLAX) view of LV (1. Interventricular septum IVS, 2 End-diastolic dimension LVIDD, 3. Posterior wall PWD). b. Mid-ventricular SAX papillary view (1. LV end-diastolic chamber area LVEDA, 2. IVS, 3. PW). c. Right ventricular focused view SC, modified from Scherrer-Crosby[210], (1. R1 mid-chamber dimension, 2. Sub-epicardial chamber area RV\textsubscript{epi}, 3. Sub-endocardial chamber area RV\textsubscript{endo}, 4. IVS). d. Short axis (SAX) right ventricular outflow tract (RVOT) view (1. Main pulmonary artery diameter PA\Phi, and locus for PWD measurement of ejection velocity time integral VTI 2. RVOT dimension R2, 3. Sub-epicardial RVOT chamber area RVOT\textsubscript{epi}, 4. Sub-endocardial RVOT chamber area RVOT\textsubscript{endo}).
5.1.2. Echocardiographic calculations

Fractional Shortening (FS) = LVIDD-LVISD/LVIDD

Fractional Area Change (FAC) = LVEDA-LVESV/LVEDA

LV volume/Stroke volume (SV) = based on geometric truncated ellipsoid model[211]

RV Free Wall Area = Epicardial area – endocardial area

RVOT Free Wall Area = Epicardial area – endocardial area

RV ejection stroke volume RVESV = PA area * VTI * HR where PA area is the cross-sectional area of the main pulmonary artery, VTI is the area beneath the PA pulsed-wave velocity waveform or velocity-time integral and HR is heart rate.

5.1.3. Strain Analysis

Real-time imaging loops of ventricular motion during the cardiac cycle were recorded for off-line analysis. The RV was imaged in the adapted SC view and the LV in the mid-ventricular SAX view. High frame rates necessitated manual tracing of epicardial and endocardial walls for 2D- strain analysis (Echopac, version 2011, GE Healthcare). Segmental and global wall motion was then analyzed.
5.1.4. Injection of Agarose Gel and Tissue Collection

Animals were euthanized using inspiratory CO₂, the heart immediately excised and injected with KCl. To correlate RV chamber size and wall thickness with echocardiographic measurement agarose gel (2%, ScientifiX, Australia) was injected into the RV chamber and left to fix at room temperature. The gel was suspended in Hank’s Balanced Salt Solution 40 mM HEPES (sHBSS). Following injection (at 10 min) the RV wall was excised from the heart, exposing the hardened agarose gel mould of the RV chamber (Figure 30). The RV free
wall was then freely dissected away from the septal wall and atrio-ventricular valves. Both the agarose gel mould and RV free wall were weighed.

5.1.5. Statistics and Analysis

Data are expressed mean (SD). Linear regression was used for correlation between echocardiographic findings and agarose gel mass, and RV wall mass at necropsy. P<0.05 was taken as significant (Graphpad Prism version 6 was used).

5.2. Results

5.2.1. Echocardiographic measurement of ventricle size and function

Left ventricle

LVIDD and LVISD were 0.71± 0.09 and 0.36± 0.07cm respectively, and using the hemisphere-cylinder model equation[211], end-diastolic chamber volume was estimated as 0.38 ± 0.09cm³. Fractional shortening was 47.21± 8.67 %, FAC measured as 65%, and calculated stroke volume 0.20 ± 0.05 ml, indicating normal systolic function.

Right ventricle

Due to the geometrical shape of the RV, the SC view imaged a larger proportion of the RV chamber than the outflow RVOT view. The SC mid-ventricular dimension (R1) was 0.42± 0.07cm, the RVOT dimension (R2) was 0.34± 0.06cm, and chamber end-diastolic area measurements were 0.36± 0.09 cm² and 0.28± 0.08cm² for SC and RVOT views respectively.
5.2.2. Correlation with necropsy

The gel mass was 0.18 ± 0.10g, the RVOT dimension (R2) was 0.34 ± 0.06cm and the mid-ventricular dimension (R1) was 0.42 ± 0.07cm for SC and RVOT views respectively. Neither RVOT nor SC dimensions correlated with agarose gel mass (R=0.098, P =0.41 and R=0.018, P=0.73) (Figure 31A). The chamber area was 0.28 ± 0.08 cm² and 0.38 ± 0.09 cm². Both RVOT and SC chamber areas correlated with gel mass (R=0.72, P =0.02 and R=0.78, P=0.03). The RV wall mass was 0.19 ± 0.04g, and the wall area was 0.069 ± 0.017cm² and 0.084 ± 0.017cm² for RVOT and SC views respectively. Neither RVOT nor SC wall area correlated with RV wall mass (R=0.004, P =0.84 and R=0.009, P=0.77) (Figure 31C).

RV chamber dimension R1 and R2 had no correlation (p= 0.41 and 0.73 respectively), whereas RVOT and SC chamber area did correlate with gel mass, (p=0.002 and 0.03 respectively, Figure 31B). Echocardiographic RV wall area, 0.08±0.02 and 0.07±0.02 cm² for SC and RVOT views respectively, corresponded to a total RV free wall mass of 0.19±0.04g.
at necropsy. There was no correlation between wall mass and RV wall area in either view, $R^2 = 0.004$ and 0.009, $p= 0.84$ and 0.77 respectively (Figure 3C).

![Figure 31. Correlation of RV chamber dimensions with gel mass from necropsy findings](image)

Comparisons between (A) agarose gel mass and right ventricle dimensions, (B) agarose gel mass and right ventricle chamber area, and (C) right ventricle wall mass and right ventricle wall area. N=8-10.

### 5.2.3. Right Ventricular ejection

Ejection indices of cardiac output (RV CO and LV CO) 110.6 ± 26.5 mL/min and 70.63 ± 17.3 mL/min respectively, left-ventricular fractional area change (LV FAC) 73.41 ± 9.3 % and Fractional shortening (LV FS) 47.21 ± 8.7 %. The average RVESV was 0.32±0.08 mL, with estimated RV CO 111 ± 26 mL.min⁻¹. These values were consistent with cardiac output
ranges measured via echocardiography in existing literature [324]. Cardiac output was higher with direct Doppler measurement of RV ejection compared to calculated cardiac output based on LV geometrical change (110.61 ± 26.53mL/min and 70.63 ± 17.29mL/min respectively). Other indices of LV ejection, FAC and FS, were consistent in measurement (Figure 32).

![Ventricular Function](image)

**Figure 32. Assessment of ventricular function**

Right ventricle cardiac output (mL/min), left ventricle cardiac output (mL/min), left ventricle fractional area change (%), and left ventricle fractional shortening (%). Data is expressed as mean ± SD, n=12-17.

5.2.4. 2D-Strain analysis

Strain analysis was performed in both ventricles. Strain analysis of left ventricular contraction was applied in the SAX view and RV analysis applied in the SC view (Figure 33). In many animals, poor endocardial border definition of the RV limited strain analysis; however, as Figure 33 demonstrates, segmental wall motion analysis was possible in the SC modified view. This study has demonstrated 2D-strain analysis of the rodent RV is feasible, and also indicates that further investigation is necessary to define normal strain values in rodents.
Figure 33. Representative 2D-Strain measurement of ventricular contraction

(A) Mid-papillary SAX view of left ventricle with segmental wall analysis. (B) Similar measurement of right ventricular free wall contraction in modified SC view. Segmental deformation during cardiac cycle relative to aortic valve closure (AVC).
5.3. Discussion

This study has shown echocardiographic measurement of RV chamber area to correlate with RV chamber gel volume in both mid-ventricular SC and RVOT views (Figure 31B). Chamber width dimension or RV wall area however, did not correlate with gel volume or wall mass at necroscopy in these normal animals (Figure 31C). In pathological states of RV dilatation or hypertrophy, it is possible that correlation with echocardiographic measurement may become evident, as changes in dimension and area are more pronounced. The study has also demonstrated the mid-ventricular SC view to be suitable for strain analysis, since a large area of the RV free wall is easily visualized. Examination with the RVOT view provides further valuable imaging of the RV infundibulum and proximal pulmonary artery, and enables measurement of RV stroke volume using pulsed-wave Doppler. Analysis of the pulmonary spectral velocity waveform also allows measurement of the pulmonary artery acceleration time (PAAT), an index of elevated pulmonary arterial pressure in rodent models [325]. Both views are complimentary, and together; provide a focused but comprehensive assessment of right ventricular structure and function. A focus of this study was to identify a practical approach to RV assessment, particularly when transthoracic imaging is limited in small animals.

Current clinical guidelines advise the use of multiple echocardiographic views, for comprehensive assessment of right ventricular function [323]. This relates to the complex RV anatomy, involving a pyramidal-shaped chamber, with inlet and outlet portions communicating to the right atrium and main pulmonary artery. Unlike the left ventricle, the right ventricular wall comprises myofibrils predominantly oriented in a longitudinal direction, with circumferential myofibrils present only in the thinner subepicardium [326]. Consequently RV contraction is effectively longitudinal with ejection propagating from apex towards outflow tract, and rotational deformation being only a minor component of RV
contraction [327]. During RV contraction, ventricular length shortens and the base is translocated towards the apex. Analysis of this dynamic ventricular movement is used to assess both contractile function and diastolic relaxation. During systole the tricuspid annular plane descends towards the apex, which itself remains relatively stationary [328]. Tricuspid annular plane systolic excursion (TAPSE) provides an approximation of RV longitudinal contractility, and can be measured using M-mode or with pulse-wave tissue Doppler imaging (S, E’ and A’ waveforms). With this approach Linqvist et al reported RVOT fractional shortening (RVOTFS) to correlate with both RV longitudinal function as measured by TAPSE, and pulmonary arterial pressure [329]. TAPSE, however, was not performed in this study since this it is appropriately measured using the more expanded apical 4-chamber view.

2D-strain is a further analysis of cardiac motion based on the tracking of “speckle” movement within the myocardial wall during the cardiac cycle. Naturally occurring bright speckles within the myocardium act as acoustic markers for tracking, and are generated by backscatter signal during ultrasound examination [330]. Analysis is offline and based on commercial software algorithms. Strain ($\varepsilon$) is calculated from the relation $L - L_o / L_o$, where $L$ is the final length and $L_o$ the initial length of a myocardial wall speckle. Strain measures vector movement in relation to myofibril architecture: deformation is negative with longitudinal and circumferential strain, and positive for radial strain relating to wall thickening. Strain rate is the velocity of deformation, or $\varepsilon$/time between frames.

The value of LV global longitudinal strain (GLS) is evident in clinical studies, where strain deformation has an earlier prognostic value than ejection fraction, particularly in predicting major adverse cardiac events [331]. Strain analysis of the RV is similarly reported to identify early changes in RV function before maladaptive remodelling becomes evident. In patients with pulmonary hypertension for example, longitudinal strain is segmentally decreased despite normal global RV function [332]. With respect to animal models the
application of 2D-strain is emerging, particularly in the assessment of left ventricular function [322, 333, 334]. However the application of RV strain to animal models, is not previously reported, but as with clinical studies, has major potential in characterizing RV function. This study has demonstrated 2D-strain analysis of the rodent RV is feasible, and also indicates that further investigation is necessary to define normal strain values in rodents.

The use of animal models of disease has direct translational value in assessing the efficacy of potential therapeutic agents [319]. In COPD, for example, it is recognized that 40% of deaths in COPD result directly from associated cardiovascular disease and particularly from RH dysfunction [14] [335, 336]. In many patients, mild to moderate pulmonary hypertension (PH) is common [337], with mean pulmonary arterial pressure (mPAP) between 25 and 30 mmHg [338]. The adaptive response of the right ventricle to increased pulmonary pressure includes chamber dilatation, wall hypertrophy and systolic dysfunction [339]. The presence of PH in COPD is associated with a poor prognosis and reduces survival [340]. In this respect assessment of right ventricular function using 2D-echocardiography remains an important aspect of clinical management [341].

A limitation to this study was the variation in image quality between animals. Technical accuracy is dependent on anatomical recognition and orientation, which ensures standardization of echocardiographic views. Incomplete or oblique views, poor definition of endocardial or epicardial borders, as well as Doppler mal-alignment, are all potential sources of error. Our experience indicates incomplete imaging to occur in only 1 of the 15 animals. However strain analysis was particularly affected by poor border definition and high frame rates, and prevented analysis in many animals. With gel injection and RV wall dissection at autopsy, potential artifactual error was possible between animals, as well as the failure to index measurements to animal body surface area. A further limitation is that CO was not validated with ultrasound measurement of PA flow, but our values were consistent with
reported values. Transthoracic examination was well tolerated, with hemodynamic stability present during anesthesia.

In summary, we have demonstrated that examination using the modified SC and RVOT views has the potential to provide a comprehensive assessment of the RV particularly when standard 4C or 2C apical views are often suboptimal. These views enable a large section of the RV free wall to be imaged, enabling assessment of dilatation, wall hypertrophy and strain analysis. Further examination of the RVOT allows RV morphology, and contractile function to be serially assessed. In rodents 2D-strain imaging of the RV is feasible, and with appropriate study design has the potential to identify early systolic dysfunction.
Chapter 6: General Discussion
6. Major Findings

The present studies examined whether targeting the oxidant-dependent mechanisms that drive COPD and its co-morbidities had a positive impact on the pulmonary and extrapulmonary manifestations of the disease. We assessed the effectiveness of two antioxidant therapies that act on different levels of the reactive oxygen species cascade via differing mechanisms of action. In Chapter 3, we first assessed the efficacy of the Gpx-1 mimetic ebselen, exploring the impact of enhanced hydrogen peroxide reduction in an established animal model of COPD. In Chapter 4, apocynin was used to reduce superoxide production via inhibition of NOX-2 in the same in vivo model of COPD.

The 8-week cigarette smoke exposure model used in Chapters 3 and 4 was able to manifest pulmonary, systemic, and skeletal muscle characteristics of COPD in the form of lung inflammation, systemic inflammation, reduced body mass, skeletal muscle oxidative damage and skeletal muscle wasting. Although there were differences in the overall effectiveness of the two novel antioxidant compounds in this model, both significantly improved lung inflammation as measured by BALF cellularity and pro-inflammatory cytokine and chemokine expression in lung tissue. Though these findings are in accordance with the established anti-inflammatory effects of ebselen and apocynin in acute respiratory conditions [229, 298], only the therapeutic effectiveness of apocynin extended to the observed skeletal muscle wasting in mice whereas the GPX-1 mimetic ebselen did not produce the same anti-wasting effect. While this lack of effect in muscle was surprising, apocynin’s superior efficacy compared to ebselen was expected as apocynin acts earlier in the ROS cascade. As described in Chapter 1, inhibition of NOX-2 prevents superoxide generation and the subsequent formation of all other oxidants in the ROS and RNS cascades, whereas mimicking Gpx-1 only inhibits hydrogen peroxide accumulation and hydroxyl
radical formation. As most published studies exploring the use of antioxidant therapy in cachexic, sarcopenia, and skeletal muscle wasting show little to no benefit [212, 342, 343], this was the first study to show the successful inhibition of cigarette smoke-induced lung inflammation and skeletal muscle wasting in an established animal model of COPD via targeted antioxidant therapy (i.e. inhibition of NOX-2 with apocynin). This proof of concept supports the potential benefits of targeting oxidant dependent mechanisms for dual therapy to treat both pulmonary and systemic manifestations of COPD. While there are many potential targets in oxidative stress pathways, our findings present the use of apocynin to inhibit NOX-2 as a logical choice for further antioxidant research. It should be noted that the physiological functions of several NOX isoforms remain unidentified and potential unpredictable side effects of long-term NOX inhibition need to be considered [344, 345]. Therefore, apocynin may not necessarily be the best option for clinical trials until its mechanism of action in this model is fully characterised in pre-clinical studies.

Additionally, we developed and validated multiple methodologies to assess the contribution of oxidative stress to the development of comorbid skeletal muscle wasting and cardiovascular disease. In addition to the in vivo studies in Chapter 4, a pilot study was conducted to develop an in vitro model that could be used to identify the specific mechanisms driving the oxidative stress-induced alterations to skeletal muscle in COPD. By modifying the parameters used in similar studies [315, 346, 347], we successfully determined the conditions required to alter skeletal muscle morphology, promote inflammation, and induce impaired mitochondrial function in C2C12 myotubes via stimulus with hydrogen peroxide. Whilst the use of this in vitro model was not explored further in this thesis, this model was extensively used in other studies conducted by our group.
As cardiovascular comorbidities of COPD can take at least 6 months to develop in animal models of COPD [348], we required a non-invasive technique that could be used to assess the development of these manifestations in vivo without terminating the animals so longitudinal studies could be performed. In Chapter 5, an innovative method to measure changes in rodent cardiac morphology and function was developed, validated and successfully published [349]. Whilst we intended to utilize this novel methodology in a chronic smoke exposure protocol once validated, no further experiments were conducted due to time constraints. Other known comorbidities of COPD were not investigated in this thesis.

6.1. Limitations

The largest limitation to this study was the inability to combine the data from both Study A and Study B for both Chapters 3 and 4. This resulted in a reduced sample size for all parameters explored resulting in decreased statistical power. While the protocols for each study were identical in terms of handling, cigarette smoke exposure and treatment, the differences between the animal research facilities that each study was conducted in resulted in major variables that could not be ignored. The University of Melbourne animal facility used in Study A was equipped with a class II biosafety cabinet with a HEPA filter and filtered mouse cages that housed up to 12 animals concurrently. The RMIT facility on the other hand was equipped with fume cupboards and ventilated microisolation cages that housed a maximum of 6 mice.

A class II biosafety cabinet equipped with a HEPA filter is inadequate for the complete ventilation of cigarette smoke, as although they draw smoke away from the animals, only the particulate matter in the smoke is filtered and the gases are released back into the room. Fume cupboards are more suited for the removal of smoke as they exhaust fumes to an external duct and are able to remove much more volatile compounds than cigarette smoke at a much faster
rate than biosafety cabinets. This means that the animals in Study A had greater exposure, albeit passively, to the gaseous components of cigarette smoke over the 8-week periods.

In addition to this, the ventilated microisolator cages at the RMIT facility provided the animals with superior air quality and reduced cage population/density outside of smoke exposure sessions. It has been shown that increased population and cage density results in negative outcomes for animals such as reduced body weights and increased mortality [350] and while we did not see a clear correlation between cage density and smoke response, the combination of these factors may explain the discrepancies in the response of the mice to smoke exposure in the four studies described in this thesis.

In addition to the sample size issue, there was insufficient time to explore all the parameters relevant to our study. While we were able to determine the overall therapeutic effects of the novel antioxidant compounds and establish a framework for future studies, many questions and potential research avenues remain. In ideal circumstances, we would have conducted the following experiments: measure lung and serum inflammatory cytokine and chemokine levels, measure lung mRNA expression in all studies, measure other markers of oxidative stress and ROS generation in the lung and skeletal muscle, and also examine alterations to skeletal muscle morphology in mice via immunohistochemistry. Given more time, we would conduct the above experiments using the stored tissue collected during the four studies outlined in this thesis. These experiments would have provided further characterization of both the smoke exposure model and the novel antioxidants explored, and may provide further insight on the mechanisms driving the comorbidities of COPD. Lastly, we would have also conducted an in vivo study to explore the role of oxidative stress in comorbid cardiovascular disease; however, this was not viable due to the length of smoke exposure required to induce these changes.
Overall, given these limitations, it was still possible to determine that antioxidant therapy is a viable therapeutic approach for the treatment of COPD and skeletal muscle wasting in mice. The findings of this thesis provide a solid framework for future studies involving the oxidant-mediated pathways that overlap between COPD and its comorbidities.

6.2. Future Directions

While the primary findings of this thesis show clear therapeutic benefit for the use of targeted antioxidant therapy in COPD, further studies should be conducted before these compounds can be assessed in a clinical setting. To build on our findings, further research should 1) further optimise pre-clinical models of COPD and its comorbidities to identify the exact contribution of oxidative stress in skeletal muscle and the specific mechanism of action apocynin, 2) characterise the pulmonary manifestations in this model and the effect of antioxidant therapy in the lungs, and 3) assess the effect of targeting oxidant-dependent mechanisms in comorbid cardiovascular disease.

6.2.1. Optimisation of in vivo models to identify the exact role of oxidative stress in skeletal muscle wasting in COPD and validate NOX-2 inhibition

Oxidative stress has a well-defined association with COPD and comorbid skeletal muscle wasting but the exact role of oxidative stress is still unclear. Currently, most studies explored oxidative stress’ ability to promote the activation of the ubiquitin proteasome system, leading to increased protein breakdown [275, 351-355]. In contrast to these studies, we did not observe these changes in our model as we were unable to produce any alterations to the mRNA expression of the E3 ligases involved in the ubiquitin proteasome system in any of the smoke exposure studies. However, our data suggests that oxidant generation via NOX-2 is involved in the development of skeletal muscle wasting, as the inhibition of NOX-2 by apocynin prevented the smoke-induced wasting. The exact mechanism of action of oxidative
stress in COPD and comorbid skeletal muscle is still unclear and these mechanisms need to be identified and characterised in order to properly validate the use of targeted antioxidant therapy. In order to do so, the 8-week smoke exposure protocol needs to be optimised and explored further.

In Chapters 3 and 4, significant alterations to various parameters in the lungs, systemically, and in the skeletal muscle were observed, however, the specific alterations were inconsistent between the four in vivo studies conducted. Although the smoke-exposure methodology is well established [252, 253, 319, 356], these inconsistencies greatly limited our ability to interpret the data and suggests that there is room for further optimisation of the chronic smoke exposure protocol to improve reproducibility. The key parameters of the model that need to be optimised are the length of the protocol and the number of cigarettes per day. Acute smoke-exposure protocols generally have animals exposed to cigarette smoke over a 4-day period but with 9 cigarettes or more per day rather than 6 cigarettes as per the model used in this study [204, 229]. This 50% increase in the number of cigarettes used is able to produce a significant inflammatory response in the lungs of mice in a very short period of time. Chronic smoke exposure models that aim to induce manifestations of disease that take longer to develop, such as cardiovascular alterations, generally require a period of exposure ranging from 4 to 6 months rather than the 2 month exposure period of our model [357, 358]. As the smoke-induced alterations in this thesis were either inconsistent between studies or lacking in severity, an increase in the number of cigarettes and/or a lengthened exposure period may be able to provide more consistent and amplified smoke-induced alterations. With a more consistent model, we would be able to obtain more reliable results and better identify the mechanisms driving comorbid skeletal muscle wasting and the exact impact NOX inhibition has on these mechanisms. It should be noted that there are opportunity costs that need to be considered when making alterations to the protocol as it is a
manual process and a lengthened period of exposure would require increased researcher input to conduct the study. Therefore, the optimised model should be able to consistently induce significant pulmonary and muscular alterations in the shortest possible time.

6.2.2. Characterisation of the pulmonary manifestations induced by cigarette smoke exposure and the effect of targeted antioxidant therapy

We have previously shown that both ebselen and apocynin have therapeutic benefits in respiratory conditions, which is consistent with the observations in this thesis. Yet the experiments conducted in this thesis primarily focused on skeletal muscle wasting and pulmonary manifestations of disease were only considered briefly. As COPD is first and foremost a respiratory condition, a compound that aims to treat pulmonary and systemic manifestations of disease needs to have a substantial impact on the pulmonary symptoms of COPD. The mouse cigarette smoke exposure model is known to induce the pulmonary pathophysiology of COPD such as chronic inflammation, impaired lung function, airspace enlargement (emphysema), airway remodeling, goblet cell hyperplasia, mucus secretion, and lung vascular angiogenesis [118, 236, 359, 360].

While it is unfeasible to induce and assess all of the above parameters in a single study, the effect that targeting oxidant-dependent mechanisms of COPD has on several smoke-induced disease pathophysiologies should be explored. If targeted antioxidant therapy successfully attenuates the pulmonary manifestations of COPD, the exact mechanism by which oxidative stress contributes to the development of airway restriction in COPD could be identified. In addition to this, a successful outcome would present a strong case for these compounds to be translated for use in clinical settings for the treatment of both pulmonary and systemic manifestations of COPD.
Another consideration would be any potential impairment to pathogen response and/or clearance as a result of antioxidant therapy, particularly in the context of AECOPD. While we do not believe that the initial pathogen response would be impaired, it is possible that pathogen clearance may be impacted if oxidant generation is impaired and this will need to be explored further using models of AECOPD.

6.2.3. Assessment of targeted antioxidant therapy in animal models of comorbid cardiovascular disease

Chronic inflammation and oxidative stress are also associated with other comorbidities of COPD such as cardiovascular disease (CVD), osteoporosis, stroke, and even lung cancer [257]. As such, reducing both chronic inflammation and oxidative stress by targeting the oxidant-dependent of COPD that overlap with these comorbidities should produce therapeutic benefits that echo those found in this thesis.

Of particular interest to our research group is comorbid cardiovascular disease as it is the most frequently occurring comorbidity of COPD with 25-70% of patients presenting with pulmonary hypertension [164, 165]. Additionally, 40% of deaths in COPD result directly from associated cardiovascular disease and particularly from right heart dysfunction [14, 335, 336]. As discussed in Chapter 1, persistent low-grade systemic inflammation and increased oxidative stress has been associated with the development of comorbid CVD [106] and we postulated that targeting oxidative stress with a compound like ebselen or apocynin would be of great therapeutic benefit. As we were previously limited by the lack of non-invasive techniques to measure cardiovascular morphology and function in a non-terminal manner, we have yet to explore this aspect of COPD in our model.

With the development and validation of our modified echocardiographic methodology in Chapter 5, it is now possible to assess ventricular dilatation, wall hypertrophy, strain
analysis, ventricle morphology, and contractile function. While the in vivo model may need to be optimised to induce cardiovascular manifestations, it is possible that correlation with echocardiographic measurement may become evident as changes in dimension and area are more pronounced. As such, any therapeutic effects of antioxidant treatment would also be more noticeable when assessed with this technique and the non-terminal nature of the technique allows for longitudinal studies to be conducted. This will allow for the cardiovascular alterations to be assessed over a long period of time, providing valuable insight on the development of these alterations in response to smoke exposure. Therefore, targeted antioxidant therapy for the treatment of comorbid cardiovascular manifestations of COPD should be explored in preclinical models and assessed using the technique we have established in this thesis.

6.3. Conclusion

In summary, we conclude that targeting the oxidant-dependent mechanisms that overlap between COPD and its associated comorbidities is a viable therapeutic approach for the treatment of pulmonary and skeletal muscle manifestations of the disease. This is the first study to show that treatment with novel antioxidant compounds is able to reduce both smoke-induced lung inflammation and skeletal muscle wasting and dysfunction in an in vivo model of COPD. We found that the inhibition of NOX-2 with apocynin had greater efficacy than the mimicking of Gpx by ebselen, however the reason for this is still unclear. Nevertheless, these compounds have shown exciting promise as viable pharmacological options for COPD patients who respond poorly to conventional pharmacological therapies and/or pulmonary rehabilitation. Additionally, we developed an in vitro model that can be used to further explore the contribution of oxidative stress in skeletal muscle wasting and dysfunction. Lastly, we also developed and validated a technique to examine alterations to cardiovascular morphology and functions in vivo. In summary, this thesis provided further insights into the
therapeutic benefits of targeting oxidative stress for the treatment of COPD and its skeletal muscle wasting, contributed to the characterization of the novel antioxidants ebsele and apocynin and established viable methods for the identification of the mechanisms that drive the onset and development of comorbidities of COPD.


197


304. Vasudevan, S., et al., Inflammatory Phenotypes Associated with Chronic Obstructive Pulmonary Disease Increase Susceptibility to Exacerbation. Lessons from Single Cell


Appendices
## Appendix 1

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>TaqMan assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping gene</td>
<td>GAPDH</td>
<td>Mm99999915_g1</td>
</tr>
<tr>
<td>Inflammatory cytokines and chemokines</td>
<td>CXCL10</td>
<td>Mm00445235_m1</td>
</tr>
<tr>
<td></td>
<td>G-CSF</td>
<td>Mm00438334_m1</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>Mm01290062_m1</td>
</tr>
<tr>
<td></td>
<td>IL-17A</td>
<td>Mm00439618_m1</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>Mm00434228_m1</td>
</tr>
<tr>
<td></td>
<td>IL-23</td>
<td>Mm00518984_m1</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>Mm00446190_m1</td>
</tr>
<tr>
<td></td>
<td>MCP-1</td>
<td>Mm00441242_m1</td>
</tr>
<tr>
<td></td>
<td>MIP-2α</td>
<td>Mm00436450_m1</td>
</tr>
<tr>
<td></td>
<td>MMP12</td>
<td>Mm00500554_m1</td>
</tr>
<tr>
<td></td>
<td>MMP9</td>
<td>Mm00442991_m1</td>
</tr>
<tr>
<td></td>
<td>TGF-β</td>
<td>Mm01178820_m1</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Mm00443258_m1</td>
</tr>
<tr>
<td>Oxidative-stress enzymes</td>
<td>NOX2/CYBB</td>
<td>Mm01287743_m1</td>
</tr>
<tr>
<td></td>
<td>Gpx1</td>
<td>Mm00656767_g1</td>
</tr>
<tr>
<td>Atrophic factors</td>
<td>Atrogin-1/FBXO32</td>
<td>Mm00499523_m1</td>
</tr>
<tr>
<td></td>
<td>Myostatin</td>
<td>Mm01254559_m1</td>
</tr>
<tr>
<td>Hypertrophic factors</td>
<td>IGF1-Ea</td>
<td>Mm00439560_m1</td>
</tr>
<tr>
<td></td>
<td>IGF1-Eb</td>
<td>Mm00439559_m1</td>
</tr>
<tr>
<td>Mitochondrial factors</td>
<td>Nfe2l2</td>
<td>Mm00477784_m1</td>
</tr>
<tr>
<td></td>
<td>PPARGC1A</td>
<td>Mm01208835_m1</td>
</tr>
</tbody>
</table>

**Table 1. Taqman assay IDs.**

List of genes and their corresponding Taqman assay identification codes.
Chronic obstructive pulmonary disease (COPD) is an incurable global health burden and is characterised by progressive airflow limitation and loss of lung function. In addition to the pulmonary impact of the disease, COPD patients often develop comorbid diseases such as cardiovascular disease, skeletal muscle wasting, lung cancer and osteoporosis. One key feature of COPD, yet often underappreciated, is the contribution of oxidative stress in the onset and development of the disease. Patients experience an increased burden of oxidative stress due to the combined effects of excess reactive oxygen species (ROS) and nitrogen species (RNS) generation, antioxidant depletion and reduced antioxidant enzyme activity. Currently, there is a lack of effective treatments for COPD, and an even greater lack of research regarding interventions that treat both COPD and its comorbidities.

Due to the involvement of oxidative stress in the pathogenesis of COPD and many of its comorbidities, a unique therapeutic opportunity arises where the treatment of a multitude of diseases may be possible with only one therapeutic target. In this review, oxidative stress and the roles of ROS/RNS in the context of COPD and comorbid cardiovascular disease, skeletal muscle wasting, lung cancer, and osteoporosis are discussed and the potential for therapeutic benefit of anti-oxidative treatment in these conditions is outlined. Because of the unique interplay between oxidative stress and these diseases, oxidative stress represents a novel target for the treatment of COPD and its comorbidities.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

An increased burden of oxidative stress is an important feature of the pathogenesis of chronic obstructive pulmonary disease (COPD) and its associated comorbid diseases (comorbidities). Current forms of therapy for COPD are largely ineffective and the development of effective treatments for COPD has been severely hampered as the mechanisms and mediators that drive the induction and progression of chronic inflammation, emphysema, altered lung function, defective lung immunity and many extrapulmonary comorbidities are still poorly understood. What is known is that four primary mechanisms have been implicated in the pathophysiological alterations observed in COPD: oxidative stress; inflammation; protease–antiprotease imbalance; and apoptosis (Barnes, 2013, 2014; Hillas, Nikolakopoulou, Hussain, & Vassilikopoulou, 2013). Although mostly underappreciated, oxidative stress has been recognized as a central component in the pathogenesis of COPD as it can trigger and further potentiate the other three mechanisms. In this review we focus on the role of oxidative stress in the pathogenesis of COPD and its comorbidities. Given its pivotal role in the onset and development of COPD, oxidative stress may be a novel target for the treatment of COPD and its comorbidities.

I.1. Chronic obstructive pulmonary disease overview

COPD represents an increasing global burden, afflicting over 600 million people and corresponding to approximately 5% of all deaths globally (Koul, 2013). It is currently the third leading cause of death (Lozano et al., 2012). It is well known that lower/middle income countries bear most of the burden of COPD with almost 90% of COPD-related deaths worldwide taking place in these countries (Lopez et al., 2006). Although the incidence rate of COPD is likely to escalate in both developed and developing countries, COPD poses a heavier burden on the Asia-Pacific and African regions where smoking is still widespread and gradually increasing (Chan-Yeung, Ait-Khaled, White, Ip, & Tan, 2004; Adelyoe et al., 2015). Cigarette smoke is inarguably the biggest risk factor for COPD, with 90% of deaths from COPD directly attributable to smoking (Tashkin & Murray, 2009). Other risk factors include exposure to air pollutants and biomass fuels (Song, Christiani, XiaoangWang, & Ren, 2014). In addition, there is generally a long latency period between exposure to smoke and clinically-evident disease and as such, there is a high incidence of COPD in the older population and ex-smokers (Theander et al., 2014). This latency period generally lasts a number of years and by the time patients become symptomatic, the damage is already irreversible.

COPD is characterized by persistent airflow limitation and lung inflammation resulting in a progressive decline in lung function (Angelis et al., 2014). The main symptoms of the disease are chronic cough (smoker's cough), excessive mucus production and dyspnoea (particularly during exercise) (Theander et al., 2014). In addition to the pulmonary manifestation of COPD, many systemic manifestations occur in the form of comorbid diseases, such as skeletal muscle wasting, cardiac dysfunction, osteoporosis and lung cancer (Chatila, Thomashow, Minai, Criner, & Make, 2008; Barnes & Celli, 2009; Patel & Hurst, 2011). These comorbidities have been associated with increased oxidative stress and are known to affect, or are strong predictors of, the mortality of COPD patients independent of the decline in lung function (Schols, Slagen, Volovics, & Wouters, 1998; Marquis et al., 2002; Sin, Anthonisen, Soriano, & Agusti, 2006; Swallow et al., 2007).

Although COPD is a largely preventable disease; the numbers of diagnoses continue to increase resulting in ever increasing medical costs to patients, communities and governments (Lozano et al., 2012; GOLD, 2015). COPD patients generally have long hospital stays, require long-term treatments, and in addition to these medical costs, other “costs” such as an increase in days missed from work and limitations to quality of life, are important consequences of this disease (Rabe, 2007; Vestbo et al., 2013).

1.2. Acute exacerbations of chronic obstructive pulmonary disease

Patients with COPD often experience episodes of sudden worsening of symptoms, known as acute exacerbations (AECOPD). The Global initiative for Chronic Obstructive Lung Disease (GOLD) defines an exacerbation as “a change in the patient’s baseline dyspnoea, cough, and/or sputum and beyond normal day-to-day variations, that is acute in onset and may warrant a change in regular medication in a patient with underlying COPD”. These exacerbations are considered to be part of the natural progression and chronicity of the disease and as COPD progresses, exacerbations become increasingly more frequent (Hurst & Wedzicha, 2009; Mackay & Hurst, 2013). Exacerbations result in a dramatic increase in lung inflammation and are associated with increased systemic inflammation compared to stable disease and as a result, there is only an increase in oxidative stress markers (Stanojkovic et al., 2011). This contributes to the worsening of symptoms and it has been shown that following hospitalisation for AECOPD, patients have increased mortality rates (Steer, Gibson, & Bourke, 2010).

AECOPD is often triggered by respiratory infections, such as those caused by the bacteria Streptococcus pneumoniae and Influenza A virus, or acute exposure to airborne irritants (Repine, Bast, & Lankhorst, 1997; Mackay & Hurst, 2013). There are also many non-aetiologic risk factors that contribute to the frequency of exacerbations such as age, frequent past exacerbations and the presence of comorbid diseases (especially cardiovascular disease) (Laratta & van Eeden, 2014). The rates of exacerbations vary dramatically depending on the parameters used to define an exacerbation, however, rates of severe AECOPD measured by hospitalisations occur at an approximate rate of 0.5 to 3.6/person-year depending on the study cited (de Melo, Ernst, & Suissa, 2004; Seemungal, Hurst, & Wedzicha, 2009). The outcomes of AECOPD can vary from the return to near baseline spirometric parameters to respiratory failure and death. Exacerbations are the largest direct cost for the treatment of COPD due to the length of hospital stays and the frequency of the exacerbations per patient (Miravitlles, Murio, Guerrero, Gisbert, & EPOC, 2002; Miravitlles et al., 2004; Mackay & Hurst, 2013).

2. Oxidative stress in chronic obstructive pulmonary disease

2.1. What is oxidative stress?

Oxidative stress refers to the imbalance between the oxidant and antioxidant levels in favour of a pro-oxidant environment in cells and tissues (Kalyanaraman, 2013). An oxidant is a species that causes or promotes oxidation and an antioxidant is a molecule that inhibits either the formation of oxidants or inhibits oxidation itself. Oxidative stress arises from the inability of innate antioxidant mechanisms to neutralize oxidants generated endo- or exogenously resulting in an imbalance between oxidant and antioxidant factors. Consequently, the oxidants predominate and chronic oxidative stress occurs, leading to the modification of lipids, proteins, and DNA (Rahman, 2005; Biswas, Hwang, Kirkham, & Rahman, 2013). The harmful modifications caused by oxidative stress are referred to as oxidative damage.

Oxidative stress can result from increased production of oxidants (in the form of free radicals/reactive oxygen and nitrogen species) or from diminished antioxidant levels or reduced antioxidant enzyme activity (Kalyanaraman, 2013). The depletion of dietary antioxidants (e.g., vitamins E, C, and D, flavonoids and carotenoids) and micronutrients (e.g., iron, copper, zinc, selenium) can also contribute to oxidative stress as they are needed for proper functioning of antioxidant enzymes (Delles, Xiong, True, Ao, & Dawson, 2014). In chronic inflammatory conditions, such as COPD, oxidative stress primarily results from the increased production of reactive oxygen species (ROS) from exposure to toxins (e.g., cigarette smoke, infection) and continuous activation of endogenous enzymes (e.g., NADPH oxidases) (Fig. 1).
2.1.1. Chronic obstructive pulmonary disease and oxidative stress

The prolonged increase in oxidative stress is a major factor in potentiating both the airway and systemic inflammation in COPD and is known to play a key role in the onset and development of COPD and its comorbidities (Montuschi et al., 2000; Cavailles et al., 2013; Kirkham & Barnes, 2013). Direct damage occurs due to the oxidants found in cigarette smoke and from excessive levels of ROS and reactive nitrogen species (RNS) produced as a result of both pulmonary and systemic inflammation. An increase in ROS production in the airways is reflected by elevated levels of markers of oxidative stress (e.g., superoxide and malondialdehyde) in the airspaces, sputum, breath, lungs, and blood in patients with COPD (Rahman, 2005; Stanojkovic et al., 2011). These levels of oxidative stress markers are also dramatically increased during exacerbations of COPD (Antus, Harnasi, Drozdovszky, & Barta, 2014). Due to the nature of the oxidative burden and its consequences in the progression of COPD, this review is primarily focused on the role of ROS/RNS in the pathogenesis of COPD and its comorbidities, and the potential pharmacological targets related to ROS signalling.

2.1.2. Environmentally-derived reactive oxygen species

Each puff of cigarette smoke contains more than 10$^{15-17}$ oxidant/free radical molecules and over 4700 highly reactive chemical compounds, such as aldehydes and quinones, which increases the oxidant burden in smokers (Church & Pryor, 1985; Nakayama, Church, & Pryor, 1989; Pryor & Stone, 1993; Rahman, 2005, 2012; Kirkham & Barnes, 2013). The nature of ROS found within cigarette smoke varies from short-lived oxidants, such as the superoxide radical ($O_2^\cdot$) and the nitric oxide radical (NO$\cdot$), to long-lived organic radicals, such as semiquinones that can undergo redox cycling within the epithelial lining fluid of smokers for some considerable period of time (Nakayama et al., 1989; Valavanidis, Vlachogianni, & Fiotakis, 2009a, 2009b). Lung and systemic formation of protein carbonyls in response to cigarette smoke-derived lipid peroxides/carbonyls have also been implicated in the pathogenesis of COPD (Montuschi et al., 2000).

In addition to the release of oxidants, cigarette smoking is also associated with an increased amount of myeloperoxidase (MPO) in neutrophils, an oxidising factor that forms hypochlorous acid and converts tyrosine to tyrosyl radical (Bridges, Fu, & Rehm, 1985). Studies have shown a correlation between the content of MPO in neutrophils and the degree of pulmonary dysfunction observed in patients (Vaguliene, Zemaitis, Lavinskiene, Miliauskas, & Sakalauskas, 2013). In addition, studies suggest that neutrophil MPO-mediated oxidative stress plays a role in lung inflammation (Gernez, Tirouvanziam, & Chanez, 2010). It is also known that cigarette smoke can cause the activation of alveolar macrophages, which is observed in the bronchoalveolar lavage fluid (BALF) from the lungs of smokers and COPD patients but not present in non-smokers. The activation of macrophages contributes to the endogenous generation of ROS in the respiratory tract (Kirkham, Spooner, Houtu-Jones, & Calvez, 2003; Barnes, 2004).

2.1.3. Cellular derived reactive oxygen species

Cellular-derived ROS is enzymatically produced by inflammatory and epithelial cells within the lung and/or systemically as part of an inflammatory-immune response towards a pathogen or irritant (Kim et al., 2013). Production of ROS by phagocytes can be enhanced by oxidants present in cigarette smoke leading to the release of inflammatory mediators (Rahman, 2012). Several sources for ROS production exist within a cell; however, the primary ROS generator is the enzyme NADPH oxidase (NOX), which comes in various isoforms: NOX-1, NOX-2, and NOX-4 (Selemidis, Sobey, Wingler, Schmidt, & Drummond, 2008; Drummond, Selemidis, Griendling, & Sobey, 2011).
In humans, NOX-1 and -2 are significant ROS-generators which are made up of an enzyme complex which is present in phagocytic and non-phagocytic cells including epithelial cells, macrophages, and skeletal muscles (Griffith et al., 2009; Barbieri & Sestili, 2012). Although NOX is found in many cell types, it is latent in neutrophils under normal circumstances (MacNee & Rahman, 2001). Once activated, neutrophils and macrophages can generate ROS via the NADPH oxidase system, leading to further augmentation of oxidative stress in the lungs of smokers and COPD patients (Fig. 2).

NOX-generated ROS have long been recognized to play key roles in the pathogenesis of a number of diverse chronic lung disorders that result in obstructive physiology, in particular asthma, cystic fibrosis, and emphysema (Griffith et al., 2009). Mice deficient in p47phox or NOX-2 exhibit increased cigarette smoke-induced lung inflammation and emphysema despite decreased ROS production compared with control mice (Yao et al., 2008). The lung responses in p47phox- and NOX2-null mice were associated with increased production of pro-inflammatory cytokines and chemokines via a TLR4–NF-kB pathway, indicating that NOX-2 may mediate anti-inflammatory functions by restraining TLR4 activation (Yao et al., 2008). However, another group reported that p47phox-null mice have less inflammation, IL-6, keratinocyte-derived chemokine, and monocyte chemoattractant protein-1 in lung-lavage specimens after cigarette-smoke exposure compared with WT mice (Gicquel et al., 2008). The differences observed by these groups may be due to variability in lung compartment sampling, cellular distributions, and chronicity of cigarette-smoke exposure.

### 2.1.4. Reactive nitrogen species

Although increased production of ROS is the primary mechanism of oxidative stress in COPD/chronic lung diseases/acute lung diseases, there is compelling evidence to suggest that RNS also play a role in COPD (Ichinose, Sugiura, Yamagata, Koarai, & Shirato, 2000; Ichinose et al., 2003). Reactive nitrogen species include nitric oxide (NO•), a nitrogen free radical, and its derivative species such as peroxynitrite and nitrogen dioxide (Fig. 3). As with ROS, in addition to generation endogenously, RNS are present in cigarette smoke and air pollutants in the form of NO• and has many of the same harmful effects as ROS (Hasnis, Bar-Shai, Burbea, & Reznick, 2007). Endogenously, NO• is associated with a multitude of signalling pathways in mammalian physiological and pathological processes; however, in excess it too causes...
indiscriminate damage to surrounding tissues and can react with O$_2^•$ forming the even more harmful peroxynitrite radical (ONOO$^−$).

2.1.4.1. Nitric oxide. For years, NO$•$ had only been considered as a toxic, unstable free radical gas that was just a constituent of air pollutant and cigarette smoke. However, it is now known that NO$•$ can be generated endogenously in several types of cells (Bredt, 1999). This implicated NO$•$ in various physiological roles and pathways, including host defence, vascular regulation and neuronal communication (Kalyanarangan, 2013). NO$•$ has been well characterized in human biology and is perhaps the most important endogenous vasoprotective molecule in addition to its role in cardiovascular function (Moncada & Higgs, 2006; Tang & Vanhoutte, 2009; Vanhoutte, Shimokawa, Tang, & Feletou, 2009; Feletou, Kohler, & Vanhoutte, 2012). NO$•$ inhibits vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium, contributing to vascular homeostasis (Cooke, 2004; Moncada & Higgs, 2006; Tang & Vanhoutte, 2009; Vanhoutte et al., 2009; Feletou et al., 2012; Triggle et al., 2012). Individuals with hypertension, atherosclerosis and/or diabetes often show impaired NO$•$ signalling, highlighting the importance of NO$•$ with regards to comorbid cardiovascular disease (Huang, 2009) (see below).

Nitric oxide is generated by phagocytes and is biosynthesized endogenously from the amino acid L-arginine, oxygen, and NADPH by various nitric oxide synthase (NOS) enzymes (Palmer, Ashton, & Moncada, 1988). In addition to its signalling roles, NO$•$ has both pro-oxidant and antioxidant activities; excessive NO$•$ can cause direct oxidative damage, however, NO$•$ can also scaveng e circulating ROS (Joshi, Ponthier, & Lancaster, 1999; Wink et al., 2001). More importantly, O$_2^•^−$ can react directly with endothelium-derived NO$•$ forming the harmful ONOO$^−$ molecule.

2.1.4.2. Peroxynitrite. Peroxynitrite is formed by the reaction between O$_2^•^−$ and NO$•$ in vivo. The pairing of these 2 radicals results in ONOO$^−$ anion, which is not a free radical but is still a very potent oxidant. ONOO$^−$ is both an oxidising and nitrating agent and can thus damage a wide array of molecules, including DNA and proteins (Beckman, 1996; Szabo & Ohshima, 1997). The formation of ONOO$^−$ is of particular relevance to the comorbidities of COPD as its formation involves the consumption of NO$•$, reducing the bioavailability of NO$•$ for physiological processes (see Section 3.2.2).

2.1.5. Depletion of antioxidants

ROS/RNS generation, endogenously or endogenously, whether circulating in pulmonary vasculature, are scavenged by blood antioxidants and antioxidant enzymes. Accordingly, the ability to protect against the deleterious effects of oxidative stress depends greatly on the antioxidant capacity of the blood and the tissues (Rahman, Morrison, Donaldson, & MacNee, 1996; Rahal et al., 2014). Studies have shown that in addition to the increased levels of circulating oxidants, there is also an observed decrease in systemic antioxidant capacity in smokers and patients with COPD (Rahman, Swarska, Henry, Stolk, & MacNee, 2000). This is due to the saturation of lung antioxidants, plasma antioxidants and protein sulfhydrs by the excessive amounts of circulating ROS released by neutrophils and macrophages (Rahman et al., 2000). The saturation of many of these antioxidants, such as uric acid, glutathione (GSH), vitamin E, and ascorbate is also associated with the severity of COPD exacerbations (Rahman, Skwarska, & MacNee, 1997). Cigarette smoke, the main aetiological risk factor for COPD, has also been shown to irreversibly modify glutathione to glutathione conjugates in the airway epithelium resulting in antioxidant deficiency and injurious lung response (van der Toorn et al., 2007). Cigarette smoking also inhibits the protective expression of the Nr2/antioxidant response element pathway in peripheral mononuclear cells of smokers, favouring a pro-inflammatory state (Garbin et al., 2009). In addition to the antioxidant saturation, studies have also shown a decrease in anti-oxidative enzyme function in COPD patients; specifically reduced SOD and Gpx activity (Kurys, Kurys, Kuzniar, & Kieszk, 2001).

There is evidence to suggest that the anti-oxidant enzyme glutathione peroxidase-1 (Gpx-1) may have a role in regulating the inflammatory response to cigarette smoke exposure. Elevated levels of H$_2$O$_2$ are measured in the exhaled breath condensate of COPD patients, particularly during exacerbations (Deshuizien et al., 1996). There is upregulation of Gpx-1 gene expression in the lungs of smokers (Bentley, Emrani, & Cassano, 2008) and depletion of Gpx activity in COPD patients and smokers (Santos et al., 2004; Kluchova, Petrasova, Joppa, Dorkova, & Tkacova, 2007; Vibhuti, Arif, Deepak, Singh, & Qadar Pasha, 2007). With respect to reduced Gpx activity in COPD patients and smokers, erythrocyte Gpx activity was significantly lower in patients with severe COPD compared with patients with moderate COPD and there is a direct relationship between systemic Gpx activity and FEV$_1$ (Kluchova et al., 2007). In addition, Gpx activity was decreased in plasma from COPD patients and oxidative stress correlates with both lung function and body mass index in COPD (Vibhuti et al., 2007). Moreover, Gpx activity was decreased in total blood from smokers and ex-smokers (Santos et al., 2004). However, these studies did not identify the isoform of Gpx that was involved in reduced activity of Gpx.

2.2. Oxidative modifications

Oxidative stress causes a wide array of physiological and pathological consequences not necessarily limited to just COPD patients. In COPD, increased oxidative stress can cause cell damage, cell necrosis, apoptosis, autophagy, remodelling of extracellular matrix and blood vessels, endothelial dysfunction, inactivation of antiproteases, premature cellular senescence, elevated mucus secretion, steroid resistance, unfolded protein response, cell proliferation, epigenetic changes, and autoimmunity (Rahman, 2005; Rahman & Kinnula, 2012; Kirkham & Barnes, 2013). Oxidative modifications of DNA, proteins and lipids all contribute to the pathophysiology of the disease.

2.2.1. DNA

Unlike the other ROS and RNS which do not react with DNA bases or deoxyribonucleoside, the hydroxyl radical and peroxynitrite can react with both purine (adenine & guanine) and pyrimidine (cytosine & thymine), forming specific products (Cooke, Evans, Dizdaroglu, & Lune, 2003). This oxidative damage can lead to mutation of DNA bases caused by AT–GC transition and GC–AT transversion and, if left unrepaired, can result in changes in protein gene expression (Kalyanarangan, 2013). In addition to this, ROS/RNS-induced DNA damage also involves single- or double-stranded DNA breaks and DNA cross-links, DNA damage inducing either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability (Naito, Suematsu, & Yoshikawa, 2011).

2.2.2. Protein

Oxidative modifications to proteins caused by ROS/RNS include protein fragmentation, oxidation of amino acids, the formation of carbonyls, dityrosine and nitrated and chlorinated tyrosines (Berlett & Stadtman, 1997; Grimsrud, Xie, Griffin, & Bernlohr, 2008). In COPD and other inflammatory diseases, elevated levels of nitrated, chlorinated and brominated tyrosines have been detected in the tissues of patients (Kim, Mofarrah & Hussain, 2008). These oxidative changes to proteins can result in various functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, modifications in uptake by cells, and altered immunogenicity (Shacter, 2000).

2.2.3. Lipids

In the presence of ROS/RNS and oxygen, lipids undergo a chain oxidation reaction leading to peroxidation. This chain reaction is initiated by oxidants, primarily the hydroxyl radical and leads to the formation
ROS and RNS normally play roles in cell signalling and homeostasis; however, in excess they become increasingly toxic. The ROS and RNS O$_2^{-}$, ONOO$^-$, H$_2$O$_2$ and •OH, in addition to their indiscriminate oxidation of proteins, lipids and DNA (Taraseviciene-Stewart & Voelkel, 2012), can result in direct lung damage or induce an assortment of cellular responses. Additionally, these oxidants also influence the inflammatory responses in the lungs via various pathways such as the activation of transcription factor NF-κB, and transcription of pro-inflammatory mediator genes, further potentiating the inflammatory response in COPD (Gloire, Legrand-Poels, & Piette, 2006). This induces a pro-inflammatory state that drives the sustained production of more ROS, leading to a vicious cycle of oxidative stress and inflammation. The oxidative damage and modifications caused by ROS are known to precede the characteristic respiratory changes seen in COPD such as airway remodelling and emphysema (Barreiro et al., 2010). Recent studies have also shown a decrease in glucocorticosteroid receptor expression as a result of prolonged oxidative stress indicating that in a pro-oxidant environment, such as in COPD patients, steroid therapy is of limited therapeutic benefit highlighting the need for new treatments (van Eeden & Sin, 2013).

2.3. Effects of oxidative stress

ROS and RNS normally play roles in cell signalling and homeostasis; however, in excess they become increasingly toxic. The ROS and RNS O$_2^{-}$, ONOO$^-$, H$_2$O$_2$ and •OH, in addition to their indiscriminate oxidation of proteins, lipids and DNA (Taraseviciene-Stewart & Voelkel, 2012), can result in direct lung damage or induce an assortment of cellular responses. Additionally, these oxidants also influence the inflammatory responses in the lungs via various pathways such as the activation of transcription factor NF-κB, and transcription of pro-inflammatory mediator genes, further potentiating the inflammatory response in COPD (Gloire, Legrand-Poels, & Piette, 2006). This induces a pro-inflammatory state that drives the sustained production of more ROS, leading to a vicious cycle of oxidative stress and inflammation. The oxidative damage and modifications caused by ROS are known to precede the characteristic respiratory changes seen in COPD such as airway remodelling and emphysema (Barreiro et al., 2010). Recent studies have also shown a decrease in glucocorticosteroid receptor expression as a result of prolonged oxidative stress indicating that in a pro-oxidant environment, such as in COPD patients, steroid therapy is of limited therapeutic benefit highlighting the need for new treatments (van Eeden & Sin, 2013).

2.3.1. Systemic oxidative stress

Even after smoke cessation, systemic oxidative stress still persists in COPD patients. This is reflected by an elevation in circulating ROS and a depletion of antioxidants. The increased oxidative stress is likely due to the persistent low grade systemic inflammation resulting in the production of ROS systemically (Foschino Barbaro, Carpegano, Spanevello, Cagnazzo, & Barnes, 2007). This systemic oxidative stress has been implicated in various systemic manifestations of COPD, with many studies associating this increased burden of oxidative stress with skeletal muscle wasting and cardiovascular disease in COPD (Foschino Barbaro et al., 2007; Dalal, Shah, Lunacek, & Hanania, 2011; Zampetaki, Dudek, & Mayr, 2013). In addition to the links between oxidative stress in COPD and comorbid diseases, increased extrapulmonary oxidative stress is also an independent risk factor of many comorbidities such as cardiovascular disease, osteoporosis and depressive disorders in patients without COPD (Sanchez-Rodriguez, Ruiz-Ramos, Correa-Munoz, & Mendoza-Nunez, 2007; Kawada, 2012; Michel, Pulchen, & Thome, 2012).

3. Cardiovascular disease (CVD)

3.1. Cardiovascular disease overview

CVD is a broad term used to describe any disease involving the heart and/or blood vessels. The presence of CVD is very common in COPD patients and is the leading cause of morbidity and mortality in younger patients and patients with mild-to-moderate COPD (Sin et al., 2006). Comorbid CVD can manifest itself in one or more various disorders such as angina, stroke, arrhythmias, hypertrophy of the heart, and myocardial infarction, and its presence greatly reduces the survivability of COPD patients (Dalal et al., 2011).
3.1.1. Cardiovascular disease and chronic obstructive pulmonary disease links

It is well documented that COPD and CVD share many of the same risk factors, such as smoking, diet, and pre-existing hypertension. When considering these risk factors it is of significance that many, if not all, contribute to disease progression at least in part via oxidative stress. Multiple studies have shown links between COPD and classical cardiovascular risk factors such as family history of coronary heart disease and diabetes (Sidney et al., 2005; Bursi, Vassallo, Weston, Killian, & Roger, 2010; Terzano et al., 2010; Ford et al., 2012). Persistent low-grade systemic inflammation is present in both COPD and CVD and oxidative stress, which plays a major role in COPD, has also been implicated in CVD (Zampetaki et al., 2013). This indicates that the systemic inflammation and increased oxidative stress in COPD may lead to the onset and development of comorbid CVD.

In addition to this, acute inflammatory lung conditions, such as bacterial/viral infection or acute exposure to airborne irritants, are also associated with vascular dysfunction (Mills et al., 2005, 2007). Increased systemic inflammation, as a result of these lung conditions, is capable of destabilizing vulnerable plaques, inducing a prothrombotic state (Man, Van Eeden & Sin, 2012). COPD patients generally do not tolerate cardiac injury or intervention as well as healthy individuals. COPD patients with acute myocardial infarction have a five-year survival rate of 46% compared to 68% in those without COPD (Bursi et al., 2010).

3.1.2. Cardiovascular disease and acute exacerbations of chronic obstructive pulmonary disease

Although many factors have been associated with poor outcomes from AECOPD, CVD is becoming increasingly recognized as a strong predictor of in-hospital mortality. Studies have shown that over 50% of patients hospitalized for AECOPD have a high prevalence of coexisting cardiovascular disease (Stefanelli et al., 2013). Comorbid cardiovascular disease is independently associated with increased risk of AECOPD (Masunaga et al., 2003). The severity of airway obstruction is a major predictor of AECOPD, and is also an independent risk factor for cardiovascular disease (Roberts et al., 2002; Mannino & Davis, 2006; Steer et al., 2010; Mannino, 2011). There is also mounting evidence associating a high frequency of acute CVD with acute respiratory illness, such as pneumonia or AECOPD. Studies have shown that in the general population, subjects with respiratory tract infections are more likely to experience a myocardial infarction within 2 weeks of infection (Meier, Jick, Derby, Vasilakis, & Jick, 1998; Corrales-Medina, Madjid, & Musher, 2010). A retrospective review examining 24 h mortality following AECOPD hospitalisation found that approximately 60% of deaths that occurred resulted from cardiovascular causes (Pastor et al., 2013).

3.1.3. Pulmonary hypertension, hypertrophy and heart failure

There are two principal pathological features in the pulmonary vascular system common to most forms of pulmonary hypertension: excessive vasoconstriction and remodelling of the pulmonary arterial wall, which primarily occur by a mechanism of smooth muscle proliferation within the medial layer (Demarco, Whaley-Connell, Sowers, Habibi, & Delsipger, 2010). Because ROS may promote vasoconstriction, smooth muscle proliferation, and vascular remodelling, oxidative stress likely plays a critical role in many forms of pulmonary hypertension. Multiple studies have shown that 25–70% of COPD patients have pulmonary hypertension, depending on the definition used (Rasubala, Yoshikawa, Nagata, Iijima, & Ohishi, 2003; Sekine et al., 2014). In addition to this, it is estimated that 25% of patients with moderate to severe COPD develop pulmonary hypertension within 6 years if they have no hypertension at baseline (Zhai, Yu, Wei, Su, & Christiani, 2014). The pathological changes implicated in the development of pulmonary hypertension can also be seen in tissue samples of COPD patients who do not have a diagnosis of pulmonary hypertension (Weitzenblum, 1984; Santos et al., 2002).

A major feature of COPD is emphysema, contributing to airway obstruction. The destruction of alveolar walls and enlargement of airspaces results in a reduction in gas exchange (van der Toorn et al., 2007). To compensate for this, blood pressure is increased to facilitate more pulmonary blood flow leading to pulmonary hypertension. This results in increased work in the right heart and over time this increased workload can lead to concentric hypertrophy of the right ventricle (RV) (Nedyoutov, Tzortzaki, Chatziantoniou, & Siatoukas, 2012). This can lead to reduced ejection fraction, elevated end diastolic RV pressure, impedance of the right heart and, if left unchecked, can lead to heart failure. These changes can be detected in COPD patients, especially in end-stage COPD patients, using transthoracic echocardiography, which allows for the measurement of various parameters in the heart such as systolic and diastolic wall thickness, and chamber size. Both pulmonary hypertension and right heart failure are associated with an increase in morbidity and mortality in COPD patients, independent of the decline in lung function (Stone, Machan, Mazer, Casserly, & Klinger, 2011).

3.2. Oxidative stress in cardiovascular disease

The presence of oxidative stress in the form of increased ROS/RNS formation (e.g., O$_2^•$–, ONOO$^–$) has been observed both clinically and in animal models of CVD (Schnabel & Blankenberg, 2007; Afanas’ev, 2011). Although implicated in CVD, the cause–effect relationship of oxidative stress with any of the different cardiovascular diseases has yet to be established. The increased generation of ROS due to impaired mitochondrial reduction of molecular oxygen, secretion of ROS by inflammatory cells, endothelial dysfunction, auto-oxidation of catecholamines, as well as exposure to radiation or air pollution can drive the oxidative stress in cardiac and vascular myocytes (Dhalla, Temsah, & Netticadan, 2000). In addition to the increased oxidant production, depression in the antioxidant reserve has also been implicated in CVD. These typically phenolic antioxidants (e.g., vitamin E) act as a protective mechanism in cardiac and vascular myocytes, and their reduced levels appear to be due to both the saturation by excess ROS/RNS and/or changes in gene expression. The harmful effects caused by ROS/RNS in cardiovascular tissues are mainly due to ability of the oxidant species to drive changes in subcellular organelles (e.g., mitochondria), reduce NO bioavailability, and induce intracellular Ca$^{2+}$–overload (Dhalla et al., 2000).

3.2.1. Cardiovascular generation of reactive oxygen species/reactive nitrogen species

As stated earlier, in addition to phagocytising cells, NOX-dependent ROS-generation has been observed in numerous non-phagocytising cells, though at a lower level. In the cardiovascular system these include vascular smooth muscle cells (VSMCs), endothelial cells, adventitial and cardiac fibroblasts and cardiomyocytes (Cave, Grieve, Johar, Zhang, & Shah, 2005). Normally, these cells continuously generate low levels of ROS even in the absence of external stimuli, and ROS derived from vascular NOX act as second messengers in VSMC signalling. Over long periods of time, vascular NOX complexes only produce low levels of O$_2^•$–, with much of it generated intracellularly where it participates in cell signalling (compared to phagocyte O$_2^•$– which is generated extra-cellularly) (Fisher, 2009). When stimulated however, there is a significant increase in NOX-driven ROS production by these cells. It is important to recognize that both vascular and phagocytic NOX play an important role in superoxide production as phagocytes can infiltrate cardiovascular tissues and facilitate the functional and structural alterations observed in CVD (Matoba & Egashira, 2011).

3.2.2. Oxidative stress in the pathogenesis of cardiovascular disease

Damage to the endothelium is the initiating step in CVD. This damage can expose endothelial cells, along with the underlying cell layers, to the deleterious effects of the inflammatory process, which can ultimately lead to the formation of atherosclerotic lesions. Cellular oxidative stress caused by excess ROS/RNS production is considered to be
intrinsic to atherosclerotic lesion formation (Vogiatzi, Toussoulis, & Stefanadis, 2009). Exogenous factors contributing to oxidative stress such as smoking and comorbid diabetes also contribute to vascular oxidative stress and are strong risk factors for CVD (Fearon & Faux, 2009). Cigarette smoke has been associated with the down-regulation of key exogenous and endogenous antioxidants such as vitamin C (ascorbic acid), carotene, Gpx and SOD (Tsuchiya et al., 2002; Agnihotri et al., 2009). This can lead to dysfunction in endothelial cells, monocytes and VSMCs as well as mitochondrial damage. In addition to this, DNA damage can be caused by oxidised lipids and this may also contribute to the dysfunction of endothelial cells, VSMCs, T lymphocytes and macrophages (Madamanchi & Runge, 2007).

Oxidative stress has also been associated with the apoptosis or programmed cell death of cardio myocytes (Singal, Khaiper, Palace, & Kumar, 1998). The loss of myocytes via apoptosis has been observed in the infarct regions of myocardium from patients that have suffered from heart failure or a myocardial infarction (MI) (Krijnen et al., 2002). Both in vitro studies and in vivo animal model studies found that apoptosis occurs in response to cardiovascular complications, such as MI, and chronic pressure overload (Fiorillo et al., 2005). The common factor in all of these conditions is the generation of oxidative stress and oxidative stress is known to play a role in the initiation of apoptosis (Nagata et al., 2003). Additionally, the apoptosis of myocytes is inhibited by antioxidants such as vitamin E and SOD, implicating ROS/RNS in the pathological pathways of CVD (Kumar, Lou, & Singal, 2002). Although studies have implicated a role for ROS/RNS in CVD related apoptosis, the exact contribution of oxidative stress in the loss of myocardial function and heart failure remains to be established.

3.2.3. Nitric oxide in cardiovascular disease

The role of nitric oxide (NO) in vascular homeostasis and signalling has been well characterized. As stated earlier, NO plays a pivotal role in the maintenance of vascular tone and vasoactivity. In contrast to this distinct role in cell physiology, NO can also contribute to CVD pathology. Under certain conditions eNOS (the endothelial isoform of the nitric oxide synthase) becomes uncoupled from a NO to an O₃− producing state (Montezano & Touyz, 2012). The excess O₃ can react with NO+ resulting in the formation of excessive ONOO−. This reaction reduces the bioavailability of NO, leading to cardiovascular dysfunction and reduced endothelial vasoregulatory capacity (Kolluru, Bir, & Kevil, 2012). ONOO− has also been shown to promote the uncoupling of eNOS, propagating the increased ONOO− formation and decreased NO bioavailability (Cassuto et al., 2014).

3.3. Current treatments and antioxidant therapies

The current treatments for comorbid CVD are the same treatments used for CVD independent of COPD, such as β receptor blockers, ACE inhibitors, or angiotensin receptor blockers. However, the benefit of these drugs in COPD patients with CVD is conflicting. Some studies have found that these treatments may be detrimental for COPD patients, worsening the pulmonary symptoms of the disease (Nojiri et al., 2014). However, others have shown that when given to COPD patients with and without comorbid COPD, these treatments have shown notable benefits with regards to airway symptoms (Huang et al., 2013). This may be due to the fact that both ACE inhibitors and angiotensin receptor blockers have displayed pleiotropic antioxidant effects in addition to their anti-hypertensive effects (Munger, 2011). Thus, this presents a unique opportunity for dual action therapy using CVD-specific treatments and antioxidants, and obviously warrants further research.

CVD and COPD also overlap with regards to their non-pharmacological management. Cardiovascular modifying factors such as physical activity and diet modification are reported to decrease hospital readmission for COPD (McLachlan, Hambly, Almsherqi, El Oakley, & McGuire, 2006). The recognition of the importance of oxidative stress in CVD has led to the fervent use of antioxidants in the treatment and prevention of the disease, however, the results of prospective, randomized clinical trials have been generally disappointing (Myung et al., 2013; Ye, Li, & Yuan, 2013). In contradiction, studies have shown that antioxidant therapy is beneficial in non-comorbid hypertension, atherosclerosis, ischaemic heart disease, cardiomyopathies and congestive heart failure (Dhalla et al., 2000). It should be noted that almost all antioxidant clinical studies only explored the effectiveness of traditional scavenging antioxidants such as vitamin E.

With regards to antioxidant treatments, the protective role of exercise in preventing oxidative stress is noteworthy. Acutely, it is known that exercise causes oxidative stress (Fisher-Wellman & Bloomer, 2009). However, exercise also leads to longer-term activation and enhanced synthesis of antioxidants and antioxidant enzymes (e.g., SOD, Gpx-1), as well as decreasing oxidant production (Gonzalez, Marquina, Rondon, Rodriguez-Malaver, & Reyes, 2008). This may explain the links between prolonged exercise and the beneficial effects to both COPD and CVD patients, and highlights the potential benefits of targeting oxidative stress in comorbid CVD.

Overall, it appears that targeting oxidative stress with antioxidant enzyme modifying treatments may have great potential in COPD and comorbid CVD by providing beneficial effects with regards to both the pulmonary and cardiovascular aspects.

4. Skeletal muscle wasting

4.1. Skeletal muscle wasting overview

Skeletal muscle wasting, also referred to as cachexia or skeletal muscle atrophy, occurs in approximately 20 to 40% of all COPD patients (Schols et al., 1998; Congleton, 1999; Maltais et al., 2014). Muscle wasting is characterized by a marked decrease in skeletal muscle mass, an increase in proportion of type I muscle fibres in the diaphragm and type 2 muscle fibres in the periphery, and associated with low exercise capacity and skeletal muscle weakness. Skeletal muscle wasting in COPD is a strong predictor of mortality, independent of decline in lung function (Schols et al., 1998; Marquis et al., 2002; Swallow et al., 2007). Although present in a large population of patients, the prevalence of muscle wasting can only be approximated as there are no simple techniques to measure muscle mass. Knowing this, the actual prevalence and extent of muscle wasting in the COPD population are likely underestimated. This is because all the data is extrapolated from body weight measurements and lean body mass, an index of muscle mass, may be reduced despite the preservation of total body weight. This is further supported by the fact that patients have a proportionally greater reduction in thigh muscle cross-sectional area compared to reduction in body weight (Marquis et al., 2002). This indicates that a preferential loss of muscle tissue exists in emaciated patients with COPD (Kim et al., 2008).

Skeletal muscle wasting, although not a directly fatal condition, reduces health-related quality of life and decreases survivability for COPD patients. The main feature of comorbid skeletal muscle wasting is a reduction in fat free mass (FFM) and is associated with weaker peripheral muscles, impaired functional status, as well as poor health-related quality of life (Debigare, Cote, & Maltais, 2001; Mathur, Brooks, & Carvalho, 2014). In COPD, the reduction in muscle mass is proportional to that of the reduction in strength indicating that the remaining contractile apparatus may be functionally preserved. However, in patients chronically treated with glucocorticosteroids, it is possible for the loss of strength to be disproportional to the reduction in muscle mass.

Deteriorations in FFM have also been described following acute exacerbations. These COPD patients also experience a reduction in exercise capabilities, with skeletal muscle alterations contributing to limitations in exercise in addition to pulmonary dysfunction (Wust & Degens, 2007). It should be noted that the strength of the quadriceps is a key determinant of exercise tolerance in COPD. This is explained by the influence that muscle strength has on the perceived leg effort.
required during exercise, which is considered to be the main limiting symptom in 40–45% of patients with COPD (Debigare & Maltais, 2008).

4.1. Mechanisms of skeletal muscle dysfunction

The pathophysiological interaction between COPD and alterations in skeletal muscle tissue is poorly understood and represents an important gap in knowledge of the disease. Skeletal muscle wasting in COPD is multifactorial in nature with several of these factors likely interacting. Many factors (e.g., inflammation, oxidative stress, and poor nutrition) can initiate or enhance alterations in skeletal muscle, such as change in fibre type phenotypic expression and regenerative defects in peripheral muscles of patients with COPD (Maltais et al., 2014). In this review, multiple risk factors will be briefly discussed along with a more indepth discussion regarding the role of oxidative stress and ROS.

4.1.1. Smoking. Cigarette smoking or exposure to other airborne irritants is unlikely to be the primary mechanism involved in skeletal muscle wasting in COPD, as seen in several studies where patients and control subjects were matched for smoking history (Maltais et al., 2014). Nevertheless, smoking does have some effect on muscle biology and it may predispose patients to the development of skeletal muscle dysfunction (De Paepe et al., 2008; Rinaldi et al., 2012). Smoking is also associated with skeletal muscle weakness in otherwise healthy individuals (Seymour et al., 2010; Barreiro et al., 2011; van den Borst et al., 2011).

4.1.1.2. Disuse. Peripheral (or limb) muscle dysfunction in COPD has been partly attributed to a reduction in physical activity, or “deconditioning.” In general, the disuse of muscle can lead to many of the features and alterations of skeletal muscle in COPD patients: muscle weakness, muscle atrophy, loss of type I fibres, decreased cross-sectional area of muscle fibres, reduced oxidative enzyme activity, reduced capillary-to-fibre ratio, early lactate release, reduced rate of phosphocreatine synthesis after exercise and altered redox status (Booth & Golliwick, 1983; Coyle, Martin, Bloomfield, Lowry, & Holloszy, 1985; Larsson & Ansved, 1985; Polkey & Moxham, 2006). In healthy adults these changes are fully reversible in response to training and increased exercise; however, full recovery is unusual in COPD patients (Troosters, Gosselin, & Decramer, 2000; Polkey & Moxham, 2006, 2011; Man, Kemp, Moxham & Polkey, 2009).

4.1.1.3. Inflammation. A low body mass index (BMI) has been linked to systemic inflammation in COPD patients. As seen in Fig. 5, inflammation plays a key role in the activation of protein breakdown in skeletal muscle. Inflammation results in the production of key cytokines, such as IL-8, that can generate an array of cellular responses. This includes the induction of the ubiquitin proteasome (UbP) system through the transcriptional activities of NF-kB and FOXOs, apoptosis, and macroautophagy, all of which have been linked to the muscle atrophy (Kandarian & Jackman, 2006). In COPD patients, there is a lack of evidence with regards to inflammation in skeletal muscle during stable disease (Gosker et al., 2003; Montes de Oca et al., 2005); however, increased inflammation is seen during periods of exacerbation (Spruit et al., 2003; Yende et al., 2006). Because of this, the role of inflammation as the key event for the development of skeletal muscle dysfunction in COPD is still widely debated.

4.1.1.4. Hypoxia. In humans (Hoppeler et al., 1990) and animals (Magalhaes et al., 2005) muscle mass decreases under hypoxic conditions. COPD patients that have low arterial O₂ and reduced O₂ delivery tend to have lower body mass than those with normal levels of arterial O₂ and sufficient O₂ delivery (Semenza, 2009). Hypoxia may be a factor driving changes in limb muscle tissue as hypoxia can induce downstream effects leading to the activation of the UbP system and reduced myogenesis (Caron, Theriault, Pare, Maltais, & Debigare, 2009).

4.2. Oxidative stress in skeletal muscle wasting

In addition to chronic inflammation, hypoxia, cigarette smoke, sepsis and an increased cost of breathing cause the increased generation of oxidants in the lungs (e.g., H₂O₂, O₂•⁻, MDA) (Rahman, 2005). It is suggested that, in addition to inflammatory mediators, these oxidants can

---

**Fig. 5.** Regulation of muscle mass relies on various hypertrophy and atrophy signalling pathways. In skeletal muscle wasting, there is an imbalance between these anabolic and catabolic processes resulting in enhanced muscle degradation as a result of protein breakdown. Myostatin (a negative regulator of muscle mass) can inhibit muscle growth. Inflammatory mediators and ROS can lead to the downstream activation of atrophy-related genes (e.g., Atrogin-1, MuRF-1) resulting in enhanced protein degradation. Inflammation and oxidative stress have also been implicated in the activation of the ubiquitin-proteasome (UbP) system. The UbP system is the primary mechanism of the protein catabolism in mammalian skeletal muscle. Refer to Maltais et al. (2014) for an exhaustive overview of the signalling pathways involved.
spill into circulation, increasing the systemic oxidative stress burden of COPD patients (Zeng et al., 2013). In in vivo models of skeletal muscle wasting in COPD, increased oxidative stress has been observed (Rinaldi et al., 2012). This increase in oxidative stress can modify muscle proteins, reducing their integrity and enhancing their degradation (Aiken, Kaake, Wang, & Huang, 2011). Direct exposure to oxidative stress from environmentally-derived oxidants, or indirect exposure via cellular-derived ROS due to inflammation, can induce proteolysis. Acute bouts of physical exercise and acute exacerbations can also increase the level of oxidative stress (Couillard et al., 2003; Karadag, Karul, Cildag, Yilmaz, & Ozcan, 2008; Stanojkovic et al., 2011). It is suggested that oxidative stress may acutely affect skeletal muscle function by inhibiting the activity of the sodium/potassium pump, sarcoplasmic reticulum function, myosin ATPase and mitochondrial respiration (Wust & Degens, 2007). In addition to these acute effects, chronic oxidative stress also contributes to muscle wasting and dysfunction in both respiratory and peripheral muscles. The increased presence of ROS associated with COPD causes an increase in the expression of UbP components leading to increased protein breakdown in skeletal muscle (Fig. 6) (Maltais et al., 2014). There is evidence that COPD patients with muscle wasting experience a more severe abnormal oxidative stress response to submaximal and maximal exercises compared to non-muscle-wasted patients with COPD (Van Helvoort et al., 2006). Although most studies focus on the increased signalling of atrophy pathways as the major driving force of comorbid skeletal muscle wasting, it should be noted that decreases in hypertrophy signalling may also contribute greatly to the pathology of the disease. Further studies are required to determine the exact contribution of these various pathways to the disease.

4.3. Current treatments and antioxidant therapies

There are currently no effective drug treatments for skeletal muscle wasting in COPD. Pharmacological treatments that have been explored include anabolic steroids, growth hormone, other growth anabolic compounds and bioactive nutrients (e.g., ghrelin, creatine) and antioxidants. Most of these treatments have only shown little to modest benefits when treating skeletal muscle wasting and weakness in COPD. As with CVD, skeletal muscle wasting and COPD also overlap with regards to their non-pharmacological management. Currently, the most potent treatment for limb muscle dysfunction is exercise training, which is already a key component for the management of COPD (Maltais et al., 2014). Some treatments such as lifestyle modifications (diet and exercise) and anabolic steroids have shown improvement in patients; however, they do not reverse the progression of muscle loss and may only increase muscle growth without substantial improvements in strength or endurance (Casaburi et al., 2004; Hansen, Gualano, Bozino’vski, Vlahos, & Anderson, 2006). Therefore, new treatments are needed as limiting muscle wasting in COPD will lead to improved quality of life and increased survival of patients. Because of its role in the pathogenesis of comorbid skeletal muscle wasting, targeting oxidative stress may be able to slow the progression or onset of the disease, giving patients an improved quality of life whilst managing their other symptoms. Furthermore, it has been reported that increasing antioxidant potential can improve muscle performance whilst attenuating muscle fatigue (Maltais et al., 2014). Further pre-clinical and clinical studies are required to determine the effectiveness of targeting oxidative stress in comorbid skeletal muscle wasting.

5. Lung cancer

5.1. Lung cancer overview

Lung cancer is one of the more fatal comorbidities of COPD. It is currently the most frequently diagnosed cancer worldwide and is the number one cause of cancer-related deaths in males (Jemal et al., 2011). Lung cancer accounts for up to 13% of all cancer diagnoses worldwide, accounting for more than one million deaths per year (Alberg &
Nomenclature, 2008; WHO, 2013). Patients with COPD have a higher risk of developing lung cancer and due to the presence of comorbid lung cancer, the survival rate of these COPD patients is drastically low (Raviv, Hawkins, DeCamp, & Kalhan, 2011). It is estimated that up to 70% of lung cancer patients have co-existing COPD, and one study found that the most common cause of death among patients with airflow obstruction was lung cancer (Lung Health Study Research, 2000). It is also known that, whilst lung cancer survival in the general population is very low, COPD patients with comorbid lung cancer have an even lower rate of survival. In one study, lung cancer patients without COPD had a 26% 3 year survival after diagnosis versus 15% in lung cancer patients with COPD (Kiri, Soriano, Visick, & Fabbri, 2010).

5.1. Lung cancer and chronic obstructive pulmonary disease links

It is well established that exposure to airborne irritants, especially those in cigarette smoke, induces both diseases. Cigarette smoke, among other airborne irritants, is an independent risk factor of both COPD and lung cancer with approximately 80% and 90% of cases associated with cigarette smoking respectively (Fathy, Hamed, Youssif, Fawzy, & Ashour, 2014). Additionally, almost 1% of COPD patients develop lung cancer yearly, which may be associated with genetic susceptibility to particulates found in cigarette smoke (Sekine, Katsura, Koh, Hiroshima, & Fujisawa, 2012). COPD is also considered to be a risk factor for lung cancer independent of smoking status. It has also been suggested that even a small reduction in airflow significantly predicted lung cancer (Wilson et al., 2008). The Multiple Risk Factor Intervention Trial reported that a 10% reduction in lung function was associated with an almost 3-times greater risk of lung cancer compared to patients without airflow limitations, after adjusting for smoking (Shaten et al., 1997). It also indicated that the lag time between smoking cessation and the beneficial effects on lung cancer development may be as long as 20 years. A separate study found that the presence of COPD increases the risk of lung cancer by up to 4.5-fold (Wang, 2013). This indicates that COPD patients have a greater risk of developing lung cancer compared to those with normal pulmonary function regardless of smoker status. On the other hand, a study looking at the prevalence of COPD in lung cancer patients, independent of age, sex, or smoking history, determined that the prevalence of pre- or co-existing COPD in recently diagnosed lung cancer cases was six-times greater than in smokers without lung cancer (Young et al., 2009). The study found that approximately 50% of newly diagnosed lung cancer patients had COPD, whereas this number was only 8% in otherwise healthy smokers. This suggests that impaired lung function may be more important than age or smoking history as a predictor of lung cancer.

Whilst the association between lung cancer and COPD has always been of great interest, the details of their various molecular pathways involved and their clinical correlates have only begun to be established. Chronic inflammation is known to induce both COPD and lung cancer by means of the release of inflammatory mediators and the excess production of ROS/RNS (Lawless, O’Byrne, & Gray, 2009; Lee, Walser & Dubinnett, 2009; Bozinovski et al., 2015). Inflammatory mediators may promote the growth of bronchoalveolar stem cells, activation of NF-κB and signal transducer and activation of transcription 3 (STAT3), which all play crucial roles in the development of lung cancer (Sekine et al., 2012). Chronic inflammation leads to increased ROS production, and increased oxidative stress has also been associated with the development of lung cancer (Barreiro et al., 2013).

5.2. Oxidative stress in lung cancer

The primary association between oxidative stress and the development of lung cancer is the oxidative modifications to DNA as a result of ROS/RNS (Cooke et al., 2003; Paz-Elizur et al., 2003). Oxidative damage to DNA by ROS and RNS has been implicated in carcinogenesis (Cooke et al., 2003). As stated earlier it is known that oxidant-induced DNA damage also involves single- or double-stranded DNA breaks and DNA cross-links, DNA damage inducing either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which have been linked to carcinogenesis (Dawane & Pandit, 2012). It should be noted that studies have found that excessive amounts of NO+ increase apoptosis in some tumour cells, whereas reduced amounts of NO+ can increase the vascularity of the tumour and protect the cells from apoptosis, particularly in lung cancer (Masri, 2010).

Due to the direct damage to DNA and the increased demand for DNA repair as a result of oxidative stress, more DNA mutations may occur. One of the most important oxidative modifications to DNA is the adduct of •OH on DNA nucleobases. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) and/or its tautomeric 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) have been widely studied and proven important mutagenic adducts to DNA and are therefore key biomarkers of oxidative stress induced carcinogenesis (Valavanidis et al., 2009a, 2009b). Mutations of 8-oxodG involve a GC→AT transversion which can modify protein transcription as stated earlier in the review. In addition to this, ROS and RNS may initiate carcinogenesis by attacking DNA nucleobases causing changes in oncogenes and tumour suppressor genes (Valavanidis, Vlachogianni, Fotakis, & Louridas, 2013). Studies found that •OH can generate various DNA phenotypes with various metastatic potentials (Malins, Polissar, & Gumselman, 1996; Valavanidis et al., 2013). It is suggested that these different phenotypes likely contribute to the diverse physiological properties and heterogeneity characteristic of metastatic cell population (Malins et al., 1996).

In addition to direct damage to DNA, ROS and RNS can indirectly damage DNA via oxidative modifications to lipids. This occurs when ROS and/or RNS react with cellular membrane phospholipids generating liperoxide radicals (LOO•) and toxic aldehydes (malondialdehyde, MDA). This results in membrane permeability and microcirculation alteration and also causes the activation of nuclear factors leading to other pro-inflammatory agents (Valavanidis et al., 2013). MDA is a naturally occurring by-product of lipid peroxidation and is a known mutagenic and carcinogenic compound. MDA reacts with DNA to form multiple adducts with mutagenic potential (Marnett, 2000). Additionally, MDA itself has also been shown to be carcinogenic (Marnett & Tuttle, 1980).

As with patients with COPD and comorbid lung cancer, patients without co-existing COPD also experience an increase in systemic oxidative stress and inflammation (Barreiro et al., 2013). These processes can cause the activation of NF-κB, which has been associated with the development of comorbid lung cancer (Chen, Li, Bai, & Lin, 2011). NF-κB activation and the subsequent actions of inflammatory-related genes may play a central role in both COPD and lung cancer. Activation of NF-κB increases the release of inflammatory mediators that can induce COPD, whilst also inhibiting apoptosis, inducing proliferation and, accelerating cancer development (Karin, 2009). The release of these inflammatory mediators can activate STAT3 which is a transcription factor with various physiological functions. Continuous activation of the STAT3 signaling pathway can potentiate pulmonary inflammation and induce adenocarcinoma formation in the lung (Takata et al., 2012). Clinically, activation of STAT3 and its downstream genes serve as biomarkers of COPD and lung cancer diagnosis and prognosis (Qu et al., 2009).

As stated previously, oxidative stress can stimulate the recruitment of macrophages and neutrophils which release various cytokines and chemokines. Released matrix metalloproteinases and ROS promote inflammation, induce apoptosis, matrix degradation, and ineffective tissue repair, resulting in enlarged airspaces (emphysema) (Belvisi & Bottomley, 2003). Bronchoalveolar stem cells may attempt to repair and replace damaged alveolar cells however, in an inflammatory environment, they can enhance carcinogenesis (Sekine et al., 2012).

5.3. Current treatments and antioxidant therapies

Treatment for lung cancer, regardless of co-existing COPD, depends on the type and severity of the cancer with the main treatments being surgery, chemotherapy and radiotherapy in varying combinations
(Byers & Rudin, 2015). It is thought that chemotherapy may have suppressive effects against oxidative stress in patients that respond well to the treatment, compared to non-responders (Gupta et al., 2010). This phenomenon may be at least partially responsible for the improved survival among responders.

As with COPD, the most effective lung cancer prevention measure is smoking cessation. Because of the long lag time between smoke cessation and beneficial effects with regards to lung cancer development, new treatments for comorbid lung cancer should be primarily focused on prevention and early detection. Early detection, prevention and intervention are critical for both COPD and lung cancer as both diseases have high mortality rates and incur severe economic burdens worldwide. Due to the strong associations and interactions between lung cancer and COPD, novel therapies for the prevention of developing comorbid lung cancer should focus on targeting pathways implicated in both diseases. Inflammation and oxidative stress go hand-in-hand in the onset and development of both of these diseases and are therefore key candidates for preventative therapy of comorbid COPD. More research is required to determine the exact contribution of oxidative stress to the pathology of lung cancer and the effectiveness of antioxidant compounds in the disease.

6. Osteoporosis

6.1. Osteoporosis overview

Osteoporosis is a progressive, metabolic bone disease that is characterized by a decrease in bone mass and density which leads to a deterioration in bone strength and an increased risk of fracture (Kanis et al., 2013). Patients with osteoporosis experience reduced bone mineral density (BMD) and deterioration of bone microarchitecture (Kanis, 2002). Additionally, patients experience alterations to the amount and variety of proteins in bone as a result of the disease (Donoso, Pino, Seit, Oses, & Rodriguez, 2015). Osteoporosis presents no actual symptoms; however the major consequence of osteoporosis is the increased risk of bone fractures as a result of the bone alterations. The key difference between normal bone fractures and osteoporotic fractures is that osteoporotic fractures occur in scenarios where healthy individuals would not normally break a bone. Therefore, these fractures are often related as fragility fractures. Generally, these fragility fractures occur in the vertebral column, ribs, hips and wrists (Adachi et al., 2003; Leslie et al., 2014).

As bone is a dynamic tissue, it continuously renews itself throughout life and this is accomplished via bone remodelling. This process is carried out by a functional and anatomic structure known as the basic multicellular unit. This structure requires the coordinated action of three major types of bone cells: osteoclasts, osteoblasts and osteocytes (Feng & McDonald, 2011). The interactions between these cells and multiple molecular mediators, including hormones, growth factors, and cytokines, drive the remodelling process (Mundy, 1993). Bone remodelling follows a time sequence that lasts approximately six months in humans and during this process, osteoclasts eliminate old and/or damaged bone which is then replaced by new bone formed by osteoblasts. Osteocytes are relatively inert cells, however, they are primarily responsible for the molecular synthesis and modification, and transduction of signals (akin to the nervous system) necessary to sustain mechanical loads (Compton & Lee, 2014). In order to prevent significant alterations to bone mass and/or mechanical strength following after each remodelling cycle, the process of bone formation and bone resorption is tightly regulated and maintained in healthy bone (Feng & McDonald, 2011). Any disturbance in the remodelling process can lead to the development of metabolic bone diseases. Oxidative stress is known to affect the remodelling process by altering the functions of both osteoclasts and osteoblasts and, as such, is associated with the pathogenesis of osteoporosis. Due to the overlap in risk factors of COPD and osteoporosis, patients with COPD have an increased incidence of osteoporosis (Jorgensen et al., 2007).

6.1.1. Osteoporosis and chronic obstructive pulmonary disease links

In COPD, patient health and treatment is primarily focused on the level of lung function and ability of oxygenation. Because of this, osteoporosis often goes undiagnosed in COPD even though COPD patients are at a higher risk of developing the disease. Osteoporosis, however, may be as equally debilitating as COPD, and can further contribute to the impaired respiratory function if the patient experiences vertebral compressions and loss of height as a result of bone degradation (Silverman, 1992; Yang et al., 2007; Masala et al., 2014). It is not clear why COPD patients often develop osteoporosis, but many of the risk factors for osteoporosis overlap with or are also associated with COPD. These risk factors include smoking, chronic inactivity, reduced weight, medication taken, poor nutrition, low antioxidant status and oxidative stress (Cielen, Maes, & Gayan-Ramirez, 2014). At a molecular level, the pathophysiology underlying the development of comorbid osteoporosis is largely unknown; however, the risk factors and causes of the disease have been well established.

Chronic treatment with glucocorticosteroids (GCS) is one of the more obvious causes of osteoporosis in COPD patients (Canalis, 2005). Although the use of GCS in the treatment of COPD is controversial due to their limited effectiveness and major side effects, they are still prescribed to patients with severe COPD and patients particularly prone to exacerbations. Most studies conclude that a beneficial effect from GCS is only observed when administered to the most severely affected patients with tendency to exacerbate (Falk, Minai, & Mosenifar, 2008; Leuppi et al., 2013; Price, Yawn, Brusselle, & Rossi, 2013). The treatment with GCS for airway impairment is associated with an increased occurrence of fractures (De Vries et al., 2007). The incidence of fractures, as a result of osteoporosis, contributes to the overall disability and mortality of patients with COPD, whilst adding to the economic burden of the disease. The increased incidence of osteoporosis is also seen in patients that are treated with GCS chronically due to other chronic diseases (McDonough, Curtis, & Saag, 2008). Although GCS use is a risk factor for the development of comorbid osteoporosis, it does not entirely account for the low bone mass and high prevalence of osteoporosis in COPD patients. In addition to chronic GCS use, inflammation has also been determined to be a major risk factor of comorbid osteoporosis.

Systemic inflammation as a consequence of COPD has been linked to the development of a myriad of comorbidities, including osteoporosis. One study showed that the increased arterial stiffness due to the severity of airway obstruction experienced by COPD patients is related to osteoporosis and various markers of systemic inflammation (Sabit et al., 2007). One such inflammatory marker is interleukin (IL)-6, a cytokine which may also contribute to the increased risk of other comorbidities. In addition to its pro-inflammatory effects, IL-6 is involved in regulation of bone turnover and has been implicated in osteoporosis development (Jhimi et al., 1990). Another study suggested that COPD is associated with increased insulin resistance (another metabolic disorder) and that this is associated with elevation of the proinflammatory marker tumour necrosis factor (TNF-α) receptor expression (Hotamisligil, 1999; Nieto-Vazquez et al., 2008). TNF-α is a documented stimulator of osteoclastic bone resorption and has been implicated in postmenopausal osteoporosis (Boyce et al., 2005). In one study examining patients awaiting pulmonary transplantation, of which 56% were COPD patients, there was a positive correlation between TNF-α receptor expression and bone turnover (Forli et al., 2008). Increased levels of the bone resorption marker 1-CTP were proportional to increased levels of TNF-α receptor II. This suggests a strong correlation between COPD induced inflammation and increased bone loss.

As stated earlier, inflammation in COPD is also implicated in skeletal muscle wasting leading to significant reductions in FFM. The increased enzyme catalytic activity in muscle wasting is also associated with bone loss. Significant changes in enzyme function, such as increased activity of alkaline phosphatase (ALP), have been identified in sera collected from osteoporosis patients (Leung, Fung, Sher, Li, & Lee, 1993). These changes have also been associated with increased bone metabolism,
6.2. Oxidative stress in osteoporosis

In addition to inflammation, there have been a multitude of studies implicating oxidative stress with an increased rate of bone loss, thus making oxidative stress a risk factor for osteoporosis (Varanasi, Francis, Berger, Papia, & Datta, 1999; Chavan, More, Mulgund, Saxena, & Sontakke, 2007; Stanojkovic et al., 2013; Cervellati et al., 2014). It is suggested that ageing and the consequential increase in ROS are responsible for bone loss (Almeida & O’Brien, 2013). Increased oxidative stress is associated with alterations in the activity and function of both osteoblast and osteoclast cells; the two major bone cells involved in the pathogenesis of osteoporosis.

6.2.1. Osteoblasts

The role of oxidative stress in osteoblasts has only been researched extensively in the past decade. As stated earlier, osteoblasts are responsible for replacing old or damaged bone with new bone. Studies found that osteoblasts can be induced to produce intracellular ROS (O$_2^*$ and H$_2$O$_2$) and the production of which can cause a decrease in ALP which can cause cell death, and this effect is partially inhibited by scavenging antioxidants (Darden et al., 1992). In high concentrations, ROS can damage osteoblast cells, preventing the normal growth and development of bone, and has been shown to induce osteoblast cell death (Lee, Lim, Lee & Yang, 2006). Additionally, H$_2$O$_2$ can modulate the activity of intracellular calcium in osteoblasts by increasing the amount of Ca$^{2+}$ released from intracellular Ca$^{2+}$ stores (Nam, Jung, Yoo, Ahn, & Suh, 2002).

6.2.2. Osteoclasts

Whilst still not well understood, the different mechanisms and pathways involved in the differentiation of osteoclasts and their ability to resorb bone are beginning to be elucidated. It is well known that ROS is involved in this process, but the extent of its contribution is still to be determined. Superoxide has been detected intracellularly in osteoclasts and at the osteoclast–bone interface, suggesting a contribution from superoxide in bone resorption (Key, Wolf, Gundberg, & Ries, 1994). It is known that osteoclastic superoxide is produced by NOX (Darden et al., 1996). H$_2$O$_2$ produced by endothelial cells associated with osteoclasts and the H$_2$O$_2$ produced by osteoclasts have been shown to increase osteoclastic activity and bone resorption, which may contribute to the increased breakdown of bone in osteoporosis (Sax et al., 1992). H$_2$O$_2$ has also been implicated in various other osteoclast functions such as osteoclast motility, differentiation of osteoclast precursors and the regulation of osteoclast formation (Steinbeck, Kim, Trudeau, Haushka, & Karnovsky, 1998). The reaction of H$_2$O$_2$ with tartrate-resistant acid phosphatase found on the surface of osteoclasts is responsible for the degradation of collagen and other proteins (Bull, Murray, Thomas, Fraser, & Nelson, 2002). Additionally, antioxidants play a role in osteoclast activity. Osteoclasts innately possess the antioxidant enzyme SOD in the plasma membrane (Steinbeck, Appel, Verhoeven, & Karnovsky, 1994). Studies found that after treating osteoclast cells with antioxidant enzymes such as SOD and catalase, ROS production is inhibited indicating that antioxidant therapy may be beneficial for patients with osteoporosis (Lee, Kim & Jang, 2014).

6.3. Current treatments and antioxidant therapies

In addition to diet supplementation with calcium and vitamin D, there is also a wide range of pharmaceuticals available for the treatment of osteoporosis. The current anti-resorptive treatments prescribed to osteoporosis patients include a number of bisphosphonates which inhibit bone resorption (MacLean et al., 2008). These vary by way of administration and are taken orally either daily, weekly, monthly or intermittently. Many other drugs are available such as, but not limited to, calcitonin and strontium renalate, both of which increase bone formation by stimulating osteoblasts, whilst reducing bone resorption via inhibiting the activity of osteoclasts. Emerging research has shown beneficial effects of anabolic treatments in osteoporosis (Neer et al., 2001). Currently, the only available pure anabolic drugs are parathyroid hormone mimetics. The use of fully human monoclonal antibodies has also been approved for osteoporosis and a number of drugs are being tested clinically for osteoporotic treatment and prevention (Lewiecki, 2011; Padhi, Jang, Stouch, Fang, & Posvar, 2011). However, unsurprisingly, there is a lack of interventional studies regarding the treatment of osteoporosis in patients with COPD.

Antioxidant treatment for osteoporosis has proven to be quite effective. The beneficial effects of antioxidants with regards to bone health and osteoporosis have also been demonstrated epidemiologically and through clinical intervention (Rao, 2013). Bearing in mind the possible adverse effects of hormonal therapy and the increasing reports regarding the side effects of bisphosphonates in the management of osteoporosis, there is a high demand for complementary and/or alternative medicine for the prevention and treatment of osteoporosis. This is especially true for COPD patients who are inherently at a greater risk of developing the disease. Due to the damaging effects of ROS on bone growth and regulation, targeting oxidative stress in COPD patients may prove to be beneficial for both COPD and osteoporosis.

7. Novel antioxidant approaches for the treatment of chronic obstructive pulmonary disease

Besides never smoking, smoke cessation is the only effective method for preventing the onset and progression of COPD. The progression of airway inflammation, increased oxidative stress, and protease burden even months/years after cessation, and nonresponsiveness to glucocorticosteroids have been documented as therapeutic challenges for the treatment of COPD (Kalyanaraman, 2013). Because of this, there is a lack of effective treatments for COPD and its comorbidities.

As stated throughout this review, tissue injury and inflammation as a result of oxidative stress are common to COPD and many of its comorbidities. A common theme throughout this review is the similarity and interplay between the pathological mechanisms of each condition and it is this similarity that provides a unique therapeutic opportunity (Fig. 6). Treatment with antioxidants may be able to progressively prevent and treat multiple diseases by suppressing the generation of ROS/RNS, neutralizing oxidants or both. Due to the overwhelming evidence implicating oxidative stress in the pathogenesis of COPD and its comorbidities, it is only logical to consider antioxidant intervention in this patient population. It is important that such interventions not only neutralize the excessive ROS and RNS generated, inhibit peroxidation of lipids, and the subsequent inflammatory response, but to also identify the source of these oxidants and inhibit their generation (Selemidis et al., 2008). Due to the imbalance in oxidants and antioxidants, this can be achieved by two approaches: increasing the endogenous antioxidant enzyme activity via enzyme modulators/mimetics, or by replenishing the depleted non-enzymatic defences through dietary or pharmacological means.

It should be noted that typical radical scavenging treatments, such as vitamin E and other dietary antioxidants, have shown minimal improvements in either COPD or the comorbidities outlined in this review. This may be due to the dose, route of administration or the specific antioxidant given and thus more research is required to determine the efficacy of these compounds with certainty. In addition, traditional antioxidants act in a "sacrificial manner," due to the fact that once they have scavenged a radical, they are essentially consumed.

Undoubtedly, the most damaging of all the ROS/RNS are the hydroxyl radical and peroxynitrite. These species are extremely reactive and
indiscriminate to the point that they basically react with the first substrate they come into contact with. Knowing this, the most effective form of protection would be to prevent their generation in the first place and this cannot be performed by traditional antioxidants (scavengers). Therefore, a pharmacological approach which inhibits the production of oxidant species is required. In addition to this, it may also prove beneficial to use combination therapy and treat patients with the pharmacological enzymatic antioxidants (e.g., NOX inhibitors) whilst also supplementing them with dietary antioxidants (e.g., vitamin E supplements).

7.1. Novel antioxidants

As shown on the ROS cascade (Fig. 2), there are 3 key enzymes that can be targeted in order to reduce oxidative stress; NOX, SOD, and Gpx/Cat. These enzymes can either be inhibited to reduce their activity (e.g., NOX inhibitor — apocynin) or amplified/mimicked to increase their activity (e.g., Gpx mimetics — ebelen). Outlined below are just some of the enzymatic compounds that may be of therapeutic benefit to COPD patients (also see Table 1).

7.1.1. NADPH oxidase inhibitors

As stated earlier, NOX-1 and -2 are the primary generators of O$_2^-$ and are responsible for the initiation of the ROS cascade (Fig. 2). NOX is a unique target in the sense that inhibiting the activity of the enzyme would not only reduce the generation of a single oxidant species but also reduce the generation of all other ROS and RNS. Inhibiting NOX would reduce O$_2^-$ production which would result in less available O$_2^-$ for the generation of H$_2$O$_2$ and ONOO$^-$, subsequently reducing •OH generation and increasing •NO bioavailability as a result.

7.1.1.1. Current studies. There are several NOX inhibitors currently being studied, however the most common is apocynin, which is a NOX inhibitor that preferentially blocks NOX-2 at low doses. It inhibits NOX by preventing the assembly of the NOX enzyme subunits, resulting in the reduced formation of NOX complexes (Selemidis et al., 2008; Drummond et al., 2011). We have shown that apocynin reduces cigarette smoke-induced lung inflammation in mice (Bernardo et al., unpublished observations). Additionally, the inhibition of NOX-2 activity ameliorates influenza A virus-induced lung inflammation, indicating that pharmacologically targeting NOX-2 may also have therapeutic potential in seasonal and possibly pandemic influenza infection (Vlahos et al., 2011; Vlahos, Stanisbax, & Selemidis, 2012). Because of this, the possible therapeutic utility of NOX-2 inhibitors may extend to AECOPD. Clinically, COPD patients treated with apocynin had reduced H$_2$O$_2$ and NO$_2^-$ in their exhaled breath concentrate compared to placebo control (Stefanska et al., 2012).

7.1.2. Superoxide dismutase mimetics

There are 3 human isoforms of SOD (1, 2, and 3), each of which can transform O$_2^-$ to H$_2$O$_2$. SOD3 (or extracellular SOD) is located in the extracellular matrix, the junctions of airway epithelial cells, the surface of airway smooth muscle, and the lining of blood vessels of the lung (Kimula & Crapo, 2003). It should be noted that smokers and COPD patients have increased sputum levels of SOD3 as an adaptive response to the increased oxidative burden (Regan et al., 2011). Additionally, polymorphisms in the SOD3 gene have been associated with emphysema but not COPD susceptibility (Sorheim et al., 2010). It is possible to enhance the conversion of O$_2^-$ to H$_2$O$_2$ by introducing SOD mimetics. This would result in the same effects caused by NOX inhibition: reduced O$_2^-$, increased NO bioavailability, however, it would also increase H$_2$O$_2$ generated. Although there is increased H$_2$O$_2$ generation, the newest class of SOD mimetics (salen complexes — “salens”) have also shown Catalase like activity, enhancing the neutralization of H$_2$O$_2$ in cells and decomposing ONOO$^-$ (Sharpe, Ollosson, Stewart, & Clark, 2002). Additionally, it has been suggested that SOD mimetics may be beneficial for the treatment of cancer in combination with traditional cancer therapies (Thomas & Sharif, 2012).

7.1.2.1. Current studies. Since the administration of exogenous SODs themselves has often proven to be problematic, a variety of innovative approaches are currently being explored, one of these being SOD mimetics. Multiple classes of SOD mimetics have been developed and each class has generally been effective in animal models of COPD. Administration of the SOD mimetic M40419 in rats treated with VEGF receptor blockers significantly decreased markers of oxidative stress in the lungs and prevented the development of emphysema (Tuder et al., 2003). In cigarette smoke-exposed rats, treatment with the SOD mimetic AEOL 10150 was found to significantly reduce BALF inflammation (Smith et al., 2002). It is known that the acute loss of SOD3 in adult mice causes death, whereas overexpression of SOD3 in animals exposed to hyperoxia reduces mortality (Folz, Abushamaa, & Suliman, 1999; Gongora et al., 2008). Additionally, administration of SOD mimetic significantly attenuated the elastase-induced emphysema in both wild-type and SOD3 knockout mice (Yao et al., 2010). This study found that SOD3 protected against oxidative fragmentation of extracellular matrix (ECM), resulting in the mitigation of lung inflammatory response and emphysema. Knowing this, it is possible that SOD3 may be more effective than other SOD isoforms for the management of COPD. Therefore, the development of pharmacological mimetics to replenish and augment SOD3 in the lung may have a therapeutic potential for the treatment of COPD/emphysema (Rahman, 2012).

7.1.3. Glutathione peroxidase mimetics

The Gpx family of enzymes, along with catalase, are responsible for the termination of the ROS cascade. There are 8 known isoforms of Gpx (1 to 8) but the most abundant isoform is Gpx-1, which is found in the cytoplasm of almost all mammalian cells and whose preferred substrate is H$_2$O$_2$ (Vlahos & Bozinoivski, 2013). The main function of Gpx is the reduction of H$_2$O$_2$ to H$_2$O and O$_2$; however, Gpx is also known to reduce lipid peroxides to their corresponding alcohols. Additionally, it has been reported that Gpx-2 is a major cigarette smoke-inducible isoform found in the lung (Singh et al., 2006). As with SOD,
it is possible to increase the reduction of H$_2$O$_2$ by introducing Gpx (or catalase) mimetics. This would result in the termination of the ROS cascade by increasing conversion of H$_2$O$_2$ to H$_2$O and O$_2$, and subsequently reduced levels of •OH and decreased levels of lipid peroxides.

7.1.3.1. Current studies. There is currently a limited amount of studies exploring the effectiveness of Gpx mimetics in COPD. When exposed to cigarette smoke, Gpx-1 knockout mice exhibited increased BALF neutrophils, macrophages, proteolytic burden, whole lung IL-17A, and MIP1α mRNA compared with WT mice (Duong et al., 2010). When treated pro-phylactically with the Gpx-1 mimetic ebselen, the cigarette smoke-induced increases in BALF macrophages, neutrophils, proteolytic burden, and macrophage and neutrophil chemotactic factor gene expression were all inhibited in both the WT and knockout mice. In addition, ebselen inhibited established BALF inflammation when administered therapeutically, suggesting that Gpx-1 mimetics may have therapeutic utility in inflammatory lung diseases where cigarette smoke plays a role such as COPD (Vlahos & Bozinovski, 2013). In addition, we have shown that ebselen caused a reduction in influenza A virus-induced lung inflammation in mice, suggesting that targeting Gpx-1 may be of therapeutic benefit for AECOPD (Yatzma et al., 2013).

Ebselen has also been shown to be protective in vivo in disease situations hallmarked by oxidative stress such as diabetes-associated atherosclerosis and cerebral ischaemia–reperfusion injury (Wong, Bozinovski, Hertzog, Hickey, & Crack, 2008). Additionally, ebselen has been used in clinical trials of acute ischaemic stroke and was found to improve the outcome of patients when administered within 24 h of stroke (Yamaguchi et al., 1998).

7.1.4. Nuclear factor erythroid 2-related factor 2 activators

In addition to the 3 enzymes in the ROS cascade, the DNA binding protein nuclear factor erythroid 2-related factor 2 (Nrf2) presents a potential target for reducing oxidative stress in COPD (Boutten, Goven, Artaud-Macari, Boczkwoski, & Bonay, 2011). Nrf2 is found in the cytoplasm of many mammalian cells and is responsible for the regulation of various antioxidants and cytoprotective genes, acting as a “master switch” for these genes. In response to oxidative stress, Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) of target genes, along with other binding factors and cofactors, resulting in the induction of stress response genes (Rahman, 2012). COPD patients have decreased levels of Nrf2 in the lungs, and may be linked to the reduced antioxidant capacity observed in patients (Nguyen, Nioi, & Pickett, 2009). In addition, Nrf2 may also help improve the clearance of bacteria by alveolar macrophages, which is particularly relevant to the prevention and treatment of AECOPD (Harvey et al., 2011).

7.1.4.1. Current studies. It is known that Nrf2 signalling is impaired in several chronic diseases, including COPD, cancer, and neurodegenerative diseases (Boutten et al., 2011; Bauer, Hill, & Alexander, 2013). The levels of Nrf2 protein are decreased in lungs of patients with COPD and one study found that Nrf2-dependent antioxidants was negatively associated with severity of COPD, suggesting that therapy directed towards enhancing Nrf2-regulated antioxidants may be a novel strategy for attenuating the effects of oxidative stress in the pathogenesis of COPD (Malhotra et al., 2008). Studies have shown increased susceptibility of Nrf2 knockout mice to C5- or elastase-induced pulmonary emphysema (Rangasamy et al., 2004; Izuka et al., 2005; Ishii et al., 2005). Compared with WT littermates, the Nrf2 knockout exhibited more pronounced inflammation and neutrophilic elastase activity in the BALF, enhanced alveolar expression of oxidative stress markers, increased numbers of apoptotic endothelial and alveolar type II epithelial cells, and decreased antioxidant and antiprotease gene expression in alveolar macrophages. Nrf2 deficiency also results in impaired alveolar type II cell growth and enhanced sensitivity to oxidants, thereby contributing to abnormal lung injury and repair (Boutten et al., 2011). Treatment with the Nrf2 activator CDDO-imidazolide was found to attenuate cigarette smoke-induced emphysema and cardiac dysfunction in mice (Sussan et al., 2009). Nrf2 appears to protect against pulmonary hyperoxia/bleomycin injury and ovalbumin challenge in mice, presumably by upregulating the transcription of lung antioxidant defence enzymes. It was also found that treatment with Nrf2 activators may be able to restore glucocorticosteroid sensitivity in COPD patients (Malhotra et al., 2011). A clinical trial of the Nrf2 activators sulforaphane is currently in progress in patients with COPD (Barnes, 2013).

Activation of Nrf2 has also been observed to have anticancer effects. Several studies have found that Nrf2-activating compounds can prevent or suppress cancer in mouse models (Sporn & Liby, 2012).

8. Concluding remarks

COPD is a major incurable global health burden and is the 4th largest cause of death in the world. Exacerbations are a common occurrence in COPD patients and contribute mainly to morbidity, death and health-related quality of life. Comorbid diseases, in particular cardiovascular disease and skeletal muscle wasting, are also common in COPD and potentiate the morbidity of COPD, leading to increased hospitalisations, mortality and healthcare costs. Current treatments have limited efficacy and fail to modify the factors that initiate and drive the long-term progression of COPD, its exacerbations and its co-morbidities. In addition, no pharmacological treatment has been shown to reduce the risk of death in COPD in prospective clinical trials. It is now evident that increased oxidative stress within the local lung microenvironment is a major driving mechanism in the pathophysiology of COPD and that it may directly influence peripheral organ (e.g., heart, skeletal muscle, brain, bone) behaviour in a ‘COPD-specific manner’. Therefore, targeting oxidant-dependent mechanisms that drive COPD and its co-morbidities may have great therapeutic potential.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors wish to thank the National Health and Medical Research Council of Australia for funding support (APP1084627). Also the authors wish to thank Mr Adrian Cajili for preparing the images.

References


obstructive pulmonary disease: the REDUCE randomized clinical trial. JAMA 309, 220–222.


RESEARCH ARTICLE

Evaluation of right heart function in a rat model using modified echocardiographic views

Ivan Bernardo†, James Wong‡, Mary E. Wlodek§, Ross Vlahos†,*, Paul Soeding‡,§,*,

1 School of Health and Biomedical Sciences, RMIT University, Bundoora, Australia, 2 Department of Cardiology, Royal Melbourne Hospital, Parkville, Australia, 3 Department of Physiology, University of Melbourne, Parkville, Australia, 4 Cardiovascular Therapeutics Unit, Department of Pharmacology, University of Melbourne, Parkville, Australia, 5 Department of Anaesthesia and Pain Management, Royal Melbourne Hospital, Grattan St, Parkville, Australia

† These authors contributed equally to this work.
* ross.vlahos@rmit.edu.au (RS); paulsoeding@bigpond.com (PS)

Abstract

Echocardiography plays a major role in assessing cardiac function in animal models. We investigated use of a modified parasternal mid-right-ventricular (MRV) and right ventricle (RV) outflow (RVOT) view, in assessing RV size and function, and the suitability of advanced 2D-strain analysis. 15 WKY rats were examined using transthoracic echocardiography. The left heart was assessed using standard short and long axis views. For the right ventricle a MRV and RVOT view were used to measure RV chamber and free wall area. 2D-strain analysis was applied to both ventricles using off-line analysis. RV chamber volume was determined by injection of 2% agarose gel, and RV free wall dissected and weighed. Echocardiography measurement was correlated with necropsy findings. The RV mid-ventricular dimension (R1) was 0.42±0.07cm and the right ventricular outflow tract dimension (R2) was 0.34±0.06cm, chamber end-diastolic area measurements were 0.38±0.09cm² and 0.29±0.08cm² for MRV and RVOT views respectively. RVOT and MRV chamber area correlated with gel mass. Doppler RV stroke volume was 0.32±0.08ml, cardiac output (CO) 110±27 ml.min⁻¹ and RV free wall contractility assessed using 2D-strain analysis was demonstrated. We have shown that modified MRV and RVOT views can provide detailed assessment of the RV in rodents, with 2D-strain analysis of the RV free wall potentially feasible.

Introduction

Animal models provide valuable insight into the pathophysiology of chronic lung disease, and enable assessment of potential therapeutics for conditions such as chronic obstructive pulmonary disease (COPD) [1–3]. Animal exposure to chronic hypoxia, monocrotaline injury or pulmonary trunk banding, have contributed greatly to understanding the relationship between lung injury and progressive dysfunction of the right ventricle (RV) [4]. In many studies echocardiography plays a major role in assessing RV function and the efficacy of therapeutic
intervention. Transthoracic views of the RV include the short-axis (SAX) mid-ventricular and aortic RV outflow views (RVOT), and the long-axis (LAX) apical 4-chamber view. Recent advances in ultrasound technology, including 2D speckle tracking or strain, have seen these modalities now being applied to clinical practice [5]. However the application of 2D-Strain in small animals is emerging, and has been recently reported for left ventricular assessment [6]. A limitation of transthoracic imaging in small animals is the effect of anatomical artifact from the thoracic cage or liver on image quality. Suboptimal imaging limits RV assessment with standard views, and can prevent the application of advanced analysis including 2D-strain imaging.

In an alternative approach, Scherrer-Crosbie et al developed a transoesophageal technique in mice, where the right ventricular wall and chamber were evaluated in the mid-right ventricular SAX view [7]. Significantly, echocardiographic findings were validated with ultrasound flow-measurement and magnetic resonance imaging (MRI). This modified RV approach is relevant to current clinical guidelines, which advocate the use of a RV-focused view over the standard four-chamber view. Optimal RV assessment occurs when imaging allows visualization of most or entire RV free wall [8].

In this study we aim to adapt the Scherrer-Crosbie mid-right ventricular (MRV) view to transthoracic examination in the rat, and compare RV assessment at the mid-ventricular SAX level, with the aortic SAX view of the right ventricular outflow tract (RVOT). The rat was the rodent of choice as it has a bigger heart than the mouse and it would therefore be easier to measure and detect small changes in size, structure and function. The RVOT view is a valuable window for assessing Doppler-derived RV ejection. In addition we plan to investigate whether the modified MRV view is suitable for 2D-strain analysis.

Methods

Studies were conducted in accordance with National Health and Medical Research Council of Australia guidelines, and approved by the University of Melbourne Animal Ethics Committee (Ethics No. 1212675). All animals were acquired from the Animal Resource Center, Perth, Australia.

Echocardiographic analysis of ventricular size and function

Fifteen male WKY rats (300-350g) were anesthetized with 2.5% isoflurane, spontaneously ventilated and placed on a heated pad in the semi-left lateral position with upright tilt, suitable for echocardiographic examination. Pulse rate, oxygen saturation and temperature were continuously monitored. After shaving, a sequential examination of the left and right ventricles was performed using a Vivid E9 with i13-L (6-14MHz) linear array transducer (GE Vingmed Ultrasound AS, Horten, Norway). Each study imaged the parasternal long axis (PLAX) and mid-papillary SAX views of the left ventricle (LV), and the modified MRV and aortic RVOT/pulmonary artery SAX view of the RV (Fig 1). LV morphology was assessed for interventricular septal (IVS) wall and posterior wall (PW) thickness, LV internal-diastolic dimension (LVIDD), LV internal-systolic dimension (LVISD), fractional shortening (FS), LV end-diastolic chamber area (LVEDA), LV end-systolic chamber area (LVESA) and fractional area change (FAC). RV chamber width was assessed at mid-chamber in the MRV view (R1) and RVOT width measured in the aortic SAX view (R2). In this view the main pulmonary artery diameter (PAa) was also measured. RV wall area was measured in both MRV and RVOT SAX views calculated from the difference between traced epicardial and endocardial areas [7]. Right ventricular ejection was measured in the aortic SAX view at the proximal main pulmonary artery (PA diameter) using 2D-Doppler and the waveform analyzed for pulmonary artery
Fig 1. Echocardiographic views used to assess cardiac function. a. Parasternal long-axis (PLAX) view of LV (1. Interventricular septum IVS, 2 End-diastolic dimension LVIDD, 3. Posterior wall dimension PWD). b. Mid-ventricular SAX papillary view (1. LV end-diastolic chamber area LVEDA, 2. IVS, 3. PW). c. Mid-right ventricular (MRV) view, modified from Scherrer-Crosby[7], (1. R1 mid-chamber dimension, 2. Sub-epicardial chamber area RV_epi, 3. Sub-endocardial chamber area RV_endo, 4. IVS). d. Short axis (SAX) right ventricular outflow tract (RVOT) view (1. Main pulmonary artery diameter PAΦ, and locus for PWD measurement of...
velocity-time integral (VTI) and time to peak ejection (PAAT). Pulmonary artery systolic pressure (PASP) was not assessed since analysis of tricuspid regurgitation jet was not performed.

Echocardiographic measurements in this study included:
- Fractional Shortening (FS) = LVIDD - LVISD / LVIDD
- Fractional Area Change (FAC) = LVEDA - LVESA / LVEDA
- LV volume/Stroke volume (SV) = based on geometric truncated ellipsoid model \[9\]
- RV Free Wall Area = Epicardial area − endocardial area
- RVOT Free Wall Area = Epicardial area − endocardial area
- RV ejection stroke volume RVESV = PA area. VTI. HR, where PA area is the cross-sectional area of the main pulmonary artery, VTI is the area beneath the PA pulsed-wave velocity waveform or velocity-time integral and HR is heart rate.

**Strain analysis**

Real-time imaging loops of ventricular motion during the cardiac cycle were recorded for offline analysis. The RV was imaged in the adapted MRV view and the LV in the mid-ventricular SAX view. High frame rates necessitated manual tracing of epicardial and endocardial walls for 2D-strain analysis (Echopac, version 2011, GE Healthcare). Segmental and global wall motion was then analyzed.

**Injection of agarose gel and tissue collection**

Animals were euthanized using inspiratory CO\(_2\), the heart immediately excised and injected with KCl. To correlate RV chamber size and wall thickness with echocardiographic measurement agarose gel (2%, ScientifX, Australia) was injected (using a 1ml syringe and 21G needle) into the RV chamber (the needle was inserted into the RV where the RV meets the apex of the heart and the gel expelled into the RV until the RV was completely saturated to the point of expansion and gel spill over outside of the heart. This occurred in 100% of animals) and left to fix at room temperature. The gel was suspended in Hank’s Balanced Salt Solution 40 mM HEPES (sHBSS). Following injection (at 10 min) the RV wall was excised from the heart, exposing the hardened agarose gel mold of the RV chamber (Fig 2). The RV free wall was then freely dissected away from the septal wall and atrio-ventricular valves. Both the agarose gel mold and RV free wall were weighed.

**Statistics and analysis**

Data are expressed mean ± standard deviation (SD). Linear regression was used for correlation between echocardiographic findings and agarose gel mass, and RV wall mass at necropsy. \(P<0.05\) was taken as significant (Graphpad Prism version 6 was used).

**Results**

Echocardiography was performed in 15 animals, and autopsy performed in 14 due to poor image quality in one animal. Animals were of similar age with mean body weight 332 ± 22 g, and heart rate 347 ± 23 bpm. Cardiac measurement of ventricular chamber size and function were within the normal range for our laboratory, and consistent with previous reports [10].

Left ventricle: LVIDD and LVISD were 0.72± 0.09 and 0.37± 0.07cm respectively, and using the hemisphere-cylinder model equation [9], end-diastolic chamber volume was
Fractional shortening was 48.25 ± 8.90%, FAC measured as 72%, and calculated stroke volume 0.20 ± 0.05 ml, indicating normal systolic function.

Right ventricle: due to the geometrical shape of the RV, the MRV view imaged a larger proportion of the RV chamber than the outflow RVOT view. The MRV chamber dimension (R1) was 0.42 ± 0.07 cm, the RVOT dimension (R2) was 0.34 ± 0.06 cm, and chamber end-diastolic area measurements were 0.38 ± 0.09 cm² and 0.29 ± 0.08 cm² for MRV and RVOT views respectively.
Correlation with necropsy: RV chamber dimension R1 and R2 had no correlation (p = 0.41 and 0.73 respectively), whereas RVOT and MRV chamber area did correlate with gel mass (p = 0.002 and 0.03 respectively, Fig 3). Echocardiographic RV wall area, 0.08 ± 0.02 cm² and 0.07 ± 0.01 cm² for MRV and RVOT views respectively, corresponded to a total RV free wall mass of 0.19 ± 0.04g at necropsy. There was no correlation between wall mass and RV wall area in either view, R 0.004 and 0.009, p = 0.84 and 0.77 respectively (Fig 3).

Correlation with necropsy: RV chamber dimension R1 and R2 had no correlation (p = 0.41 and 0.73 respectively), whereas RVOT and MRV chamber area did correlate with gel mass (p = 0.002 and 0.03 respectively, Fig 3). Echocardiographic RV wall area, 0.08±0.02 and 0.07 ±0.01 cm² for MRV and RVOT views respectively, corresponded to a total RV free wall mass of 0.19±0.04g at necropsy. There was no correlation between wall mass and RV wall area in either view, R 0.004 and 0.009, p = 0.84 and 0.77 respectively (Fig 3).

Right Ventricular Ejection. The average RVESV was 0.32 ± 0.08 ml, with estimated RV CO 110 ± 27 ml/min. These values were consistent with cardiac output ranges measured via echocardiography in existing literature [11]. Cardiac output was higher with direct Doppler measurement of RV ejection compared to calculated cardiac output based on LV geometrical change (110 ± 27 ml/min and 70.63 ± 17.29ml/min respectively). Other indices of LV ejection, FAC (72.61 ± 7.92%) and FS (48.25 ± 8.90%), were consistent in measurement (Fig 4).

2D-Strain analysis. Strain analysis was performed in both ventricles. Strain analysis of left ventricular contraction was applied in the SAX view and RV analysis applied in the MRV view (Fig 5). In many rats poor endocardial border definition of the RV limited strain analysis;
Discussion

This study has shown echocardiographic measurement of RV chamber area to correlate with RV chamber gel volume in both MRV and RVOT views. Chamber width dimension or RV wall area however, did not correlate with gel volume or wall mass at necroscopy in these normal animals. In pathological states of RV dilatation or hypertrophy, it is possible that correlation with echocardiographic measurement may become evident, as changes in dimension and area are more pronounced. The study has also demonstrated the MRV view to be suitable for strain analysis, since a large area of the RV free wall is easily visualized. Examination with the RVOT view provides further valuable imaging of the RV infundibulum and proximal pulmonary artery, and enables measurement of RV stroke volume using pulsed-wave Doppler. Our data showed stroke volume to be greater (and likely more accurate) with RVOT Doppler measurement, compared to LV measurement based on geometric estimation alone. Further the RVOT view enables analysis of the pulmonary spectral velocity waveform, with measurement of the pulmonary artery acceleration time (PAAT), an index of elevated pulmonary arterial pressure in rodent models [12]. Both views are complimentary, and together, provide a focused but comprehensive assessment of right ventricular structure and function. A focus of this study was to identify a practical approach to RV assessment, particularly when transthoracic imaging is limited in small animals.

Current clinical guidelines advise the use of multiple echocardiographic views, for comprehensive assessment of right ventricular function [8]. This relates to the complex RV anatomy, involving a pyramidal-shaped chamber, with inlet and outlet portions communicating to the right atrium and main pulmonary artery. Unlike the left ventricle, the right ventricular wall comprises myofibrils predominantly oriented in a longitudinal direction, with circumferential myofibrils present only in the thinner subepicardium [13]. Consequently RV contraction is effectively longitudinal with ejection propagating from apex towards outflow tract, and
Fig 5. 2D-Strain measurement of ventricular contraction. a. Mid-papillary SAX view of left ventricle with segmental wall analysis. b. Similar measurement of right ventricular free wall contraction in MRV view. A rapid HR necessitated manual gating of aortic valve opening and closure (AVC) for segmental deformation measurement during the cardiac cycle.

https://doi.org/10.1371/journal.pone.0187345.g005
rotational deformation being only a minor component of RV contraction [14]. During RV contraction, ventricular length shortens and the base is translocated towards the apex. Analysis of this dynamic ventricular movement is used to assess both contractile function and diastolic relaxation. During systole the tricuspid annular plane descends towards the apex, which itself remains relatively stationary [15]. Tricuspid annular plane systolic excursion (TAPSE) provides an approximation of RV longitudinal contractility, and can be measured using M-mode or with pulse-wave tissue Doppler imaging (S, E’ and A’ waveforms). With this approach Linqvist at al reported RVOT fractional shortening (RVOTFS) to correlate with both RV longitudinal function as measured by TAPSE, and pulmonary arterial pressure [16]. TAPSE, however, was not performed in this study since it is appropriately measured using the more expanded apical 4-chamber view.

2D-strain is a further analysis of cardiac motion based on the tracking of “speckle” movement within the myocardial wall during the cardiac cycle. Naturally occurring bright speckles within the myocardium act as acoustic markers for tracking, and are generated by backscatter signal during ultrasound examination [15]. Analysis is offline and based on commercial software algorithms. Strain (ε) is calculated from the relation \( L - L_0 / L_0 \), where \( L \) is the final length and \( L_0 \) the initial length of a myocardial wall speckle. Strain measures vector movement in relation to myofibril architecture: deformation is negative with longitudinal and circumferential strain, and positive for radial strain relating to wall thickening. Strain rate is the velocity of deformation, or \( \varepsilon / \text{time} \) between frames.

The value of LV global longitudinal strain (GLS) is evident in clinical studies, where strain deformation has an earlier prognostic value than ejection fraction, particularly in predicting major adverse cardiac events [17]. Strain analysis of the RV is similarly reported to identify early changes in RV function before maladaptive remodelling becomes evident. In patients with pulmonary hypertension for example, longitudinal strain is segmentally decreased despite normal global RV function [18]. With respect to animal models the application of 2D-strain is emerging, particularly in the assessment of left ventricular function [6, 19, 20]. However, the application of RV strain to animal models is not previously reported, but as with clinical studies, has major potential in characterizing RV function. This study has demonstrated 2D-strain analysis of the rodent RV is feasible. However, we are aware that limitations do exist. Firstly, strain analysis was suboptimal in many animals due to poor endocardial border definition and may be specific to our current ultrasound technology. Secondly, the MRV represents a “hybrid SAX” view with measurement of circumferential, radial (or longitudinal) wall deformation components being dependant on RV myofibril orientation, relative to the ultrasound beam. Further investigation of strain analysis using the MRV view is required to define normal values in rodents.

The use of animal models of disease has direct translational value in assessing the efficacy of potential therapeutic agents [3]. In COPD, for example, it is recognized that 40% of deaths in COPD result directly from associated cardiovascular disease and particularly from right heart (RH) dysfunction [21–24]. In many patients, mild to moderate pulmonary hypertension (PH) is common [25], with mean pulmonary arterial pressure (mPAP) between 25 and 30 mmHg [26]. The adaptive response of the right ventricle to increased pulmonary pressure includes chamber dilatation, wall hypertrophy and systolic dysfunction [27]. The presence of PH in COPD is associated with a poor prognosis and reduces survival [28]. In this respect assessment of right ventricular function using 2D-echocardiography remains an important aspect of clinical management [29].

A limitation to this study was the variation in image quality between animals. Technical accuracy is dependent on anatomical recognition and orientation, which ensures standardization of echocardiographic views. Incomplete or oblique views, poor definition of endocardial
or epicardial borders, as well as Doppler mal-alignment, are all potential sources of error. Our experience indicates incomplete imaging to occur in only 1 of the 15 animals. However strain analysis was particularly affected by poor border definition and high frame rates, and prevented analysis in many animals. With respect to gel injection and RV wall dissection at autopsy, a potential error was the failure to index measurements to animal body surface area. A further limitation in methodology is that CO was not validated with ultrasound measurement of PA flow. However our values were consistent with reported values. Transthoracic examination was well tolerated, with hemodynamic stability throughout anesthesia.

In summary, we have demonstrated that examination using the MRV and RVOT views has the potential to provide a comprehensive assessment of the RV particularly when standard 4C or 2C apical views are often suboptimal. These views enable a large section of the RV free wall to be imaged, enabling assessment of dilatation, wall hypertrophy and strain analysis. Further examination of the RVOT allows RV morphology, and contractile function to be serially assessed. In rodents 2D-strain imaging of the RV is feasible, and with appropriate study design has the potential to identify early systolic dysfunction.

Supporting information
S1 Text. Echocardiography spreadsheet containing raw data.
(XLSX)

Acknowledgments
We thank Ms Chandra Lennie (GE Healthcare) and Mr Andrew Jefferies for technical support, Mr Adrian Cajili for designing the diagrams in Fig 1, and NHMRC Australia (grant 1084627) for funding.

Author Contributions
Conceptualization: James Wong, Ross Vlahos, Paul Soeding.
Data curation: Ivan Bernardo, James Wong, Mary E. Wlodek, Ross Vlahos, Paul Soeding.
Formal analysis: Ivan Bernardo, James Wong, Ross Vlahos, Paul Soeding.
Funding acquisition: Ross Vlahos.
Investigation: Ivan Bernardo, James Wong, Mary E. Wlodek, Ross Vlahos, Paul Soeding.
Methodology: Ivan Bernardo, James Wong, Mary E. Wlodek, Ross Vlahos, Paul Soeding.
Resources: Ross Vlahos.
Supervision: Ross Vlahos, Paul Soeding.
Writing – original draft: Ivan Bernardo, James Wong, Mary E. Wlodek, Ross Vlahos, Paul Soeding.
Writing – review & editing: Ivan Bernardo, James Wong, Mary E. Wlodek, Ross Vlahos, Paul Soeding.

References


Appendix 4

Contact details for this correspondence:
Faye Bulled
Animal Ethics Officer
Office for Research Ethics and Integrity (OREI)

Telephone: [Redacted]
Email: [Redacted]

In reply please quote Ethics ID 1413348.1

11 November 2014

Associate Professor Ross Vlahos
Department of Pharmacology and Therapeutics
The University of Melbourne

Dear A/Prof Vlahos

New Research Application

Ethics ID: 1413348.1

Title: Identification of novel therapeutics for Chronic Obstructive Pulmonary Disease.

Thank you for your response to queries raised by the Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee at a meeting held on Tuesday 7 October 2014 to consider the above-named application.

On behalf of the Committee the Chair has approved the application. Please see overleaf, Summary Details for Approved Animal Ethics Project and Conditions of Approval.

Please do not hesitate to contact me if you have any queries.

Yours sincerely,

[Redacted]

Faye Bulled
Animal Ethics Officer
Summary Details for Approved Animal Ethics Project

**TITLE:** Identification of novel therapeutics for Chronic Obstructive Pulmonary Disease.

**ETHICS ID:** 1413348.1

**START DATE:** 1st November 2014  
**EXPIRY DATE:** 1st November 2017

**RESPONSIBLE AEC:** Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology

**SCIENTIFIC PROCEDURES PREMISES LICENCE:** Pharmacology (048)

**PRIMARY CONTACT:** A/Prof Ross Vlahos

**PROJECT SUPERVISOR:** A/Prof Ross Vlahos

**OTHER INVESTIGATORS:**  
Ivan Bernardo  
A/Prof Steven Bozinovski  
Chantal Donovan  
Mr Huei Seow  
Mr Selcuk Yatmaz

**Animals Approved:**

<table>
<thead>
<tr>
<th>Category</th>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Sex</th>
<th>Age</th>
<th>Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory Animals</td>
<td>Mouse</td>
<td>BALB/c</td>
<td>M/F</td>
<td>7 weeks</td>
<td>2268</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>IL-17 deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>tPA deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>uPA deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>NOX-4 deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>Gpx-1 deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>NOX-2 deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Mouse (Genetically Modified)</td>
<td>NOX-1 deficient</td>
<td>M/F</td>
<td>7 weeks</td>
<td>240</td>
</tr>
</tbody>
</table>

**Conditions of Approval**

Any amendment proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

The AEC must be notified of:
1. any adverse incidents involving animals used in the project and the steps taken in response
THE UNIVERSITY OF MELBOURNE
ANIMAL ETHICS COMMITTEE

APPLICATION FOR APPROVAL TO AMEND AN APPROVED RESEARCH PROJECT

1. ADMINISTRATION DETAILS

<table>
<thead>
<tr>
<th>SPPL:</th>
<th>Pharmacology (048)</th>
<th>ETHICS ID:</th>
<th>1112185.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC:</td>
<td>Anatomy &amp; Neuroscience, Pathology, Pharmacology, and Physiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROJECT SUPERVISOR &amp; DEPARTMENT:</td>
<td>A/Prof Ross Vlahos Pharmacology And Therapeutics</td>
<td>PRIMARY CONTACT &amp; DEPARTMENT:</td>
<td>A/Prof Ross Vlahos Pharmacology And Therapeutics</td>
</tr>
<tr>
<td>TITLE:</td>
<td>Identification of novel therapeutics for Chronic Obstructive Pulmonary Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APPROVAL DATE:</td>
<td>27 March 2014</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2 AMENDMENT SUMMARY

'Yes' answers in this section indicate amendments have been made to that section of the following application:

**Project Overview - SPPL or Title?**
No

**Risk Management/Permits?**
No

**Project Summary - Outline, Aims and Design?**
No

**JUSTIFICATION FOR THE USE OF ANIMALS**

**Potential Benefits or Impact?**
No

**Repeated Studies/Use of Animals?**
No

**The 3Rs - Replacement, Reduction, Refinement?**

Any request for more animals or revisions to project design will require an answer to this section in addition to reconsideration of all remaining Steps under the category of Refinement, Animals Requested, Transport and Location, Project Description, Personnel, Monitoring and Fate of Animals.

No

**Animals Requested?**
No

**Transport and Location?**
No

**Project Description?**
No

**PERSONNEL?**

List here names of any new person being added and/or existing person being removed and a brief reason for doing so. Then complete all changes in Personnel Step.

Yes - I wish to add Dr Michelle Hansen, Dr Samantha Passey, Dr Thippadey Khau, Ms Victoria Austin, Mr Ivan Bernardo and Mr Hoai Buu (Bruce) Ngo to this application. The proposed start date is 24 March 2014. Ms Austin and Mr Ngo are new Honours students that have joined our groups this year. Mr Bernardo is a new Master of Biomedical Science student who has joined my group this year. I have formed new collaborations with Drs Hansen, Passey and Khau.

**Monitoring or Management of Emergencies?**
No

**Fate of the Animals?**
2. PROJECT SUMMARY

BACKGROUND, AIMS AND OUTLINE

2.1 Provide a brief discussion of the background of the project and describe the progress of the project to date.

Chronic obstructive pulmonary disease (COPD) is a lung disease that afflicts millions of people worldwide and there is currently no effective therapy to treat people with this debilitating disease. Patients with COPD are also prone to respiratory infections (commonly called acute exacerbations of COPD - AECOPD) that cause an accelerated decline in lung function, hospitalisation and even death. These respiratory infections consist of bacteria and viruses that get into the lungs of people with COPD. People with COPD find it extremely difficult to fight off these respiratory infections. We have developed mouse models of COPD that replicate the features of human disease (AEEC #s 02084, 02085, 05097, 05107, 06199, 0810912). These models involve exposing mice to low concentrations of the bacterial wall component lipopolysaccharide (LPS) shown to be involved in exacerbations of COPD (to replicate the features of acute lung inflammation), low concentrations of cigarette smoke (to replicate the features of human COPD) and a mouse-adapted version of the influenza virus which is involved in acute exacerbations of human COPD (to replicate the features of acute exacerbations of COPD). Thus, we will be using three models to mimic acute lung inflammation (LPS), COPD (cigarette smoke) and acute exacerbations of COPD (Influenza). We have shown that LPS, low concentrations of cigarette smoke and Influenza cause inflammatory cells to enter the lungs. The influx of inflammatory cells and other cell types in the lungs release growth factors which cause even more inflammatory cells to enter the lungs leading to mucus secretion and blockage of the airways. In addition, we have discovered that these inflammatory cells release substances (called proteases) that cause irreversible destruction of the lungs.

2.2 State the aims of the project.

The aim of this project is to use the mouse models of lung inflammation, COPD and AECOPD to determine the processes responsible for the development of COPD and thus develop novel therapies to treat COPD. In particular, we will block the production and actions of various mediators we believe contribute to the development of acute lung inflammation, COPD and acute exacerbations of COPD, discovered from our previous work. This will be achieved using possible therapeutic agents dosed via one of the following routes: oral gavage, intraperitoneal injection, intravenous injection or intranasal administration to block the actions of previously identified mediators of the disease process. The role of certain proteins identified by our previous work as having a role in the development of disease will be assessed by using neutralising antibodies, compounds which inhibit the production of factors (siRNAs) and small molecular weight compounds which inhibit the actions of factors produced in excess by the inflammatory cells in response to LPS, cigarette smoke and Influenza. The role of certain genes identified by our previous work as having a role in the development of disease will be assessed using mice that lack these genes. Identifying and treating the cause of COPD, and its exacerbations, will lead to a reduction in the severity of COPD.

2.3 Briefly outline what will happen to the animals to be used in the amended protocols. It is important to note that this section is a summary only. Expanded detail of procedures on animals is required in the Project Description.

To date we have developed mouse models of COPD and AECOPD (AEEC # 02084, 02085, 05097, 05107, 06199, 0810912). We have shown that these models replicate the relevant aspects of human COPD. Specifically, LPS, cigarette smoke and Influenza cause inflammatory cells (macrophages, neutrophils, lymphocytes) to enter the lungs, an increase in various factors (cytokines and chemokines) being released from cells in the lung which attract even more inflammatory cells to enter the lungs, an increase in lung destroying substances (proteases), an increase in cells which secrete (goblet cells), muscle wasting and emphysema (destruction of lung tissue). We now wish to further explore the role of molecules and/or signal transduction pathways involved in the development of COPD and AECOPD.
Experiment 1: Acute lung inflammation will be induced in some mice by intranasal exposure to LPS. The inflammation will cause an influx of inflammatory cells to the lung over a period between 3-72 hours. Mice will be administered with agents during the experiment within the timeframe of -24 and 72 hours of the experiment and killed at 3, 24 or 72 hours. The role of certain genes identified by our previous work as having a role in the development of acute lung inflammation will be assessed using mice that lack these genes. Thus, gene deficient mice will be treated with LPS intranasally and killed at 3, 24 or 72 hours.

Experiment 2: COPD will be induced in another set of mice by inhalation of low concentrations of cigarette smoke for a period of between 1 and 185 days simulating COPD. Briefly, mice will be placed in an 18 litre Perspex chamber where cigarette smoke will be forced into as in previous AEC application (#02085, 05097, 0810912). Mice will be administered with agents during the experiment at various times between days -7 and 185. Gene-deficient mice will also be exposed to cigarette smoke for a period of between 1 and 185 days, but will not be treated with any agents. Mice will then be killed and the lungs evaluated for inflammation and emphysema.

Experiment 3: COPD-like exacerbations will be induced in a set of smoke-exposed mice (as described in Experiment 2) followed by infection with Influenza (smoke and influenza virus will not run concurrently). The viral infection will not exceed 10 days post cigarette smoke exposure as we know from past experience that the infection has resolved by 10 days post infection. Drug administration will occur between days -7 and 185. Mice will then be killed and the lungs evaluated for inflammation and emphysema. Gene-deficient mice will not be used in these experiments.

Previous experiments have shown that there is minimal observable discomfort to the animal with these procedures and there are no complications associated with the procedures (AECC #s 00176, 02084, 02085, 05097, 05107, 06199, 0810912). However, in the event the animals show signs of distress they will be euthanized by an overdose of anaesthetic (see attached Intervention Criteria Sheet).

Mice will be treated with one of the following agents which block the actions of specific molecules; immuno-modulatory test agents, antibodies or small molecular weight compounds that block the actions of specific molecules to determine if the severity of the disease can be reduced. The routes of administration include intraperitoneal injection, intravenous injection, subcutaneous injection and intranasal administration. At the conclusion of the experimental protocol, the animals will be killed by an overdose of anaesthetic. The lungs will be washed (bronchoalveolar lavage) for total cell number and then removed and examined for inflammatory changes. Biochemical indices of inflammation will also be measured in fluid obtained from the lungs. In addition, gene deficient mice will be exposed to LPS and cigarette smoke and the inflammatory response (or lack thereof) monitored.

JUSTIFICATION FOR THE USE OF ANIMALS

2.4 Explain the significance and the potential benefit of the proposed project.

The studies outlined in this proposal will contribute significantly to our understanding of the mechanisms underlying COPD and exacerbations of COPD (a sudden worsening of the condition usually in response to lung infections such as bacteria (LPS) or virus (Influenza). The potential benefit of the proposed project is that it may identify therapeutic targets to treat people with this debilitating disease.

2.5 Will the degree of pain or distress experienced by animals be the same as outlined in the original project.

The potential impact to animals will be mild. We have previously shown that treatment with LPS or exposure to low doses of cigarette smoke and influenza cause mild lung inflammation and minimal distress to the animal. This study induces a mild lung inflammation that, in general, correlates with GOLD standard 1, which is the lowest level of the four levels of disease severity of COPD. Based on our extensive previous experience, these mice do not show behavioral or neurological levels of distress (Chen, H, Am. J. Respir. Crit. Care Med., 2006) and mice that have been exposed to cigarette smoke for up to 6 months in one study (Hansen et al Proceedings of American Thoracic Society, 2005) do not show excessive weight loss or loss of appetite, and do not show signs of distress or display avoidance behavior. If anything, mice smoked for longer periods of time appear calm and quiet during the smoke exposure and return to normal eating/drinking behavior immediately after exposure. A monitoring checklist (see attachment), will be used to ensure comprehensive and regular monitoring of the animals, and any animals that show signs of distress (as outlined in the monitoring and intervention checklists) will be euthanized immediately by an overdose of anesthetic. At the conclusion of the experiments animals will be killed by an overdose of anesthetic. Our staff is highly experienced in these procedures.
2.6 Does this project duplicate work that has been carried out previously? If so, please explain why it is necessary to duplicate the work.

No

2.7 Have any of the animals been the subject of a previous research or teaching activity? If yes, provide AEC Register Number/s of the other project/s, describe what was done to the animals previously, and justify their use in this project.

No

REPLACEMENT

3. ALTERNATIVES

3.1 Have alternatives that totally or partially replace the use of animals been incorporated into this project? If no, identify potential alternatives and explain why they are unsuitable for use in this project. If yes, please describe what alternatives are to be used in this project.

No - The complex inflammation that triggers COPD reactions cannot be modeled using computers, nor can cell culture techniques address questions involving integrated pathophysiology. The experiments proposed in this application cannot be ethically performed in humans. The genes and proteins that regulate inflammation in the mouse are highly conserved in humans making the findings we obtain directly relevant to understanding human disease. Other members of the laboratory (Drs Steven Bozinovski and Rosa Gualano) have cell culture systems where certain cells known to be involved in COPD (eg epithelial and macrophages) are exposed to cigarette smoke extract. These studies have provided us with valuable information but are limited as they cannot replicate in vivo biology and only deals with the water-soluble components of cigarette smoke. Please note that we have designed our experiments so as to limit the number of animals used whilst obtaining maximum information.

REDUCTION

4. ANIMALS REQUESTED

<table>
<thead>
<tr>
<th>APPROVED NUMBER OF ANIMALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type/Species</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Mouse</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Type/Species</td>
</tr>
<tr>
<td>------------------------------</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
</tr>
</tbody>
</table>

NOTE: refer to section 6.2 for details of genetically modified and/or cloned animals

5. JUSTIFICATION FOR NUMBER OF ANIMALS REQUESTED

5.1 Provide an explanation for the number of animals being requested. This explanation should be based on statistical analysis and/or other considerations in the experimental design (e.g., multiple time points, quantity of tissue required). Where appropriate, present the numbers in table form.

The group size of 12 per group (8 [biochemistry] + 4 [histology]) has been based on power calculations to ensure a maximum likelihood of detecting real differences in parameters between control and treated mice based on historical variances in our laboratory for similar protocols (AEEC # 02084, 02085, 05097, 05107, 06199, 0810912). The experiments have been designed to extract the maximum information from as few mice as possible. While the animal numbers listed in the attached document (Animals Requested_AEC#1112185.doc) seem large, each experiment has been designed to produce definitive outcomes preventing possible repeats of experiments.

5.2 To reduce animal use, would the animals or their tissues be suitable for use in another project at the end of your experiment/s? Identify the suitable project, if known.

No - We have designed the experiments in order to gain the maximum amount of information about the underlying mechanisms of COPD and potential therapeutic benefits of inhibiting the mediators we have discovered from our previous studies to be involved in acute lung inflammation, COPD and AECOPD. Therefore most of the tissues from the animals will be collected for subsequent analysis. In addition, many of the animals used will have been treated with compounds or will be from genetically-modified mice and will be unsuitable for additional experimentation.

REFINEMENT
6. USE OF ANIMALS

6.1 Justify your choice of animal (species/strain/sex/age).

The genes and proteins that regulate inflammation in the mouse are highly conserved in humans making the findings we obtain directly relevant to understanding human disease. In addition, gene modification technology in the mouse will be used in this application to extend the information gained from the microarray studies in the same species. Moreover, mice are commonly used for these types of experiments due to the accessibility of the mouse genome, ready availability of commercial antibodies and other immunological reagents and the investigators have extensive experience in this species. Balb/c mice (7 weeks of age) were chosen for this series of experiments as the models being utilized in this study were developed in these mice. We also would like to conduct experiments on the various knockout mice as we have learned that removing the genes listed in the application will potentially prevent inflammation and COPD compared to the WT counterparts.

6.2 Genetic Modification or Cloning of Animals.

This project does involve the use or production of genetically modified animals. Summary details as given are listed at the end of this application.

This project does not involve the use or production of cloned animals.

6.3 Transport

6.3.1 Will animals need to be transported from the source location/s to the location where they will be held for this project?

Yes - For those animals that are not bred in the BRF, they will be transported from the source location (eg ARC, Monash Institute of Medical Research, Monash University, Ludwig Institute for Cancer Research) in transport boxes with sufficient access to food and water. The animals will be weight matched into groups and allowed to acclimatise in their micro-isolator cages for a period of a week before experimentation starts.

6.3.2 Where will procedures be performed? If animals need to be transported from where they are housed to where the procedures are carried out, provide details of transport and acclimatisation procedures.

Cigarette smoke exposure, LPS, influenza and drug administrations will be performed in the BRF. Animals will then be transported to our laboratory (N804, Level 8 of the Medical Building, North Wing) in transport boxes via the goods and services lift. Mice will then be killed and the lungs assessed for inflammation, COPD and AECOPD as described in the application.

6.4 Location & Housing

6.4.1 Where will animals be housed?

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Housing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>BALB/c</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>uPA deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>TPA deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>Gpx-1 deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>IL-23 deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>MyD88 deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>NOX-4 deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>NOX-1 deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>IL-17 deficient</td>
<td>Biological Research Facility</td>
</tr>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>NOX-2 deficient</td>
<td>Biological Research Facility</td>
</tr>
</tbody>
</table>
6.4.2 Will any animals be housed outdoors? If, contrary to the needs of the species, no shelter is provided, justify the lack of shelter.

No

6.4.3 What type of housing will be used? Describe any special housing requirements.

Mice will be housed in sterile micro-isolator cages in order to protect our mice from infection. These protect our mice from infection and while it is well established that the influenza virus strain to be used in this study is not contagious, it will further reduce the already remote risk of infection to other animals in the BRF.

6.4.4 Will any animals be housed individually? If yes, explain why, for how long and how the impact of social isolation will be minimised.

No

7. PROJECT DESCRIPTION

Once animals are obtained from the relevant source (eg ARC) they will be acclimatised to their new location for 1 week, after which animals will be subjected to one intervention to produce either acute lung inflammation, COPD and AECOPD. These interventions include LPS (acute lung inflammation), cigarette smoke exposure alone (COPD) and cigarette smoke exposure + Influenza infection (AECOPD).

Mice exposed to LPS, cigarette smoke, or smoke and Influenza will be treated with agents that block the actions of mediators that cause inflammation. The agents to be used will depend on the mediator we wish to block and its reported mode of action. Our previous studies (AEEC# 00176, 02085, 05097, 05107, 06199, 0810912) have shown that the mediators found in increased amounts in smoke-exposed lung compared to control (sham) can be blocked by using (i) small molecular weight compounds, (ii) neutralising antibodies, or iii) siRNAs. These agents will inhibit the action that a variety of inflammatory, COPD and AECOPD associated mediators have on the initial and prolonged disease state that may aid in some relief in disease. Thus we should see an inhibition of cigarette smoke-, LPS-, and cigarette smoke + Influenza-induced inflammation and associated consequential disease factors such as weight loss and muscle wasting. Depending on the experimental model and property of the mediator in question agents will be administered in a variety of ways including oral gavage, intraperitoneal injection, subcutaneous injection, intravenous injection or intranasally.

Each individual animal will receive one form of test agent by one route of administration only. Depending on the route or administration each animal will not receive more than 1 oral gavage, 2 intraperitoneal injections, 1 intranasal administration, 1 intravenous administration or 1 subcutaneous injection in a 24 hour period. Due to the nature of the agents we cannot state the exact time intervals between each procedure since many of the test agents have different pharmacokinetic profiles (the time period that the drug works).

Oral gavage: Compounds will be administered once by oral gavage in a dose range between 0.2-200 mg/kg. Animals will be restrained and a gavage tube will be used to push the head slightly upward and back to straighten the oesophagus. The tube will be positioned to the right or left of the mouth and inserted slowly while watching for the swallowing reflex. The tube will pass freely into the oesophagus, without being forced. When the desired length of insertion is achieved, the compound solution will be injected (no needle is required, volume used will be 100-200 μl per treatment). Mice will then be observed after the procedure for any signs of distress.

Intraperitoneal injections will be administered to conscious mice using a 27 gauge needle on a 1 ml syringe. The volume of compound will be no more than 300 μl.

Subcutaneous injection: Mice will be flattened slightly against a smooth stable surface and the skin around the shoulders will be lightly pinched. A small volume (maximum 200μl) of drug using a 27 gauge needle will be injected just under the pinched skin.

Intranasal administration ensures a concentrated dose of agent directly into the lung. Mice will be lightly anaesthetized with the inhalation anaesthetic methoxyflurane as described below and a small amount (50 μl) of solution containing (i)
small molecular weight compounds or (ii) neutralising antibodies or iii) siRNAs will be applied to the nostrils once per day of treatment (up to 14 days).

Intravenous administration: A final group of animals will be treated via intravascular, tail vein injections. Mice will be restrained (without sedation), using a purpose-built mouse restrainer and a small volume (50 µl) of compound will be injected, using a 30 gauge needle, into the mouse tail vein, once per day of treatment (up to 14 days).

Please note that all of these procedures will only be performed by experienced investigators.

The dose of agent to be used will be obtained from the literature or provided by pharmaceutical companies and will be used in the range known to be safe and well tolerated by mice. In designing small molecular weight compounds, each company has performed detailed pharmacokinetic analyses which will allow us to use effective doses at blood and target tissue. Since they are developmental compounds they have undergone rigorous acute (single rising dose) and 1 week protocols (sub-chronic dosing). In addition we know that the drugs are very well tolerated. Some of the companies have also developed a range of monoclonal neutralising antibodies that are safe over wide dose ranges. Monoclonal antibodies are used for larger molecular weight mediators where small molecular weight compounds do not work or have not been identified. The class and mode of action of the types of drugs to be used include: (i) chemokine receptor antagonists which block white blood cell recruitment to the lungs; (ii) PI3-kinase inhibitors which prevent inflammatory cell activation; (iii) colony stimulating factor blockers which prevent inflammatory cell signalling; (iii) protease inhibitors which prevent the actions of proteases which "eat away" the lung tissue; (iv) Monoclonal antibodies will be used to neutralise the actions of macrophage growth factors (eg GM-CSF, G-CSF), mediators which sustain neutrophilic inflammation (IL-17, IL-23), mediators which cause small airways fibrosis and inflammation (eg connective tissue growth factor) and the serine proteases uPA and tPA which cause tissue destruction; macrophage inflammatory protein-2 (MIP-2, involved in neutrophil recruitment), monocyte chemotactic protein-1 (MCP-1; involved in macrophage recruitment and activation) and tumour necrosis factor-α (TNF-α; many effects including weight loss).

Some mice will be treated with neutralising antibodies, small molecular weight compounds and siRNAs. These agents will inhibit the production (siRNAs) and action (small molecular weight inhibitors, neutralising antibodies) of macrophage growth factors and activity of kinases all of which we have found from microarray and proteomic experiments to be up-regulated by cigarette smoke and LPS. Thus we should see an inhibition of cigarette smoke and LPS-induced inflammation. These agents will be administered intranasally to ensure direct delivery into the lung.

Experiment 1: Lipopolysaccharide-induced inflammation

Lipopolysaccharide (LPS) will be used to trigger mild lung inflammation. Bacteria have been shown to be involved in human exacerbations of COPD. LPS is a bacterial cell wall component that induces inflammation in the lung, we propose to treat animals with LPS following cigarette smoke exposure to model AECOPD. Mice will be lightly anaesthetized by the inhalation anesthetic methoxyflurane by placing them in a small plastic tub (about 0.5 litre) where around 1 ml of methoxyflurane is placed on a wad of cotton wool and then 5 layers of gauze are placed on top to prevent direct contact with the anesthetic. The mouse is put into the tub and a timer started, it takes around 2 minutes for anesthesia to develop, as assessed by loss of the “righting reflex” when the tub is gently rocked. The mouse is treated with LPS by gently pipeting 50 µl of LPS (10 µg LPS, diluted in saline) onto the nostrils using a 0-200 µl pippette. Anaesthetizing the animals allows the experimenter to treat the animal quickly and effectively as they are immobilised and as mice are obligate nose breathers the saline containing LPS will be distributed uniformly throughout the lung. We have routinely used methoxyflurane for the last 15 years for this reason. In addition, there are studies showing that inhaled isoflurane inhibits LPS-induced lung inflammation in mice (Marcias et al 2010 Inhaled Isoflurane In Mice Ameliorates LPS- and Ventilator-Induced Lung Injury; Reutershan et al. 2006 Protective Effects of Isoflurane Pretreatment in Endotoxin-induced Lung Injury) and we have recently found that inhaled isoflurane inhibits cigarette smoke-induced lung inflammation. For these reasons, inhaled isoflurane cannot be used in our experiments. Animals are then placed in a recovery box for approximately 5 minutes and make a full recovery. Animals will be killed by an overdose of the anaesthetic (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg) between 3 and 72 hours after LPS administration because we have shown that there is significant accumulation of macrophages (a critical cell involved in exacerbations of COPD) within these time points. Death will be ensured by opening of the chest cavity. We have previously shown (AECC #00176) that 10 µg LPS administered intranasally causes mild inflammation with minimal observable discomfort to the animal. However, in the event the animals show signs of distress (animals will be monitored daily using attached monitoring and intervention checklists) such as loss of body weight (more then 10% over a 24 hour period), lethargy, piloerection, hunched appearance, loss of appetite and loss of grooming, they will be immediately killed by an overdose (intraperitoneal injection) of anaesthetic (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg). Death will be ensured by opening of the chest cavity. Similar experiments will also be performed in genetically modified mice to determine the importance of defects of certain genes in the development of COPD.
Mice are put into an 18 litre plastic container connected to a 3-way tap where cigarette smoke will be pumped. A cigarette is connected to the tap (using a pipette tip) along with a 60 ml syringe, which is then used to push smoke from the lit cigarette into the chamber. It takes ~3 min to smoke 1 cigarette using a constant rate of 10 sec to draw up the syringe barrel. The box lid is left on for another 12 min and then removed for 5 min of fresh air. This is done 3 times in succession 3 times per day, ie the maximum smoke exposure is 9 cigarettes per day over at least an 8 hour period. Assay for carboxyhaemoglobin in blood has shown that smoke inhalation in mice is similar to mild human smoking, and histology has shown that we are not causing acute lung injury. Over a 14 day smoke protocol, mice lose around 7% of their body weight but there is no reduction in activity and weight is rapidly regained once smoking stops. 4 days of smoke produces many features of COPD, including an increase in numbers of key types of white blood cells in the lung and increased lung protease activity. Previous experiments (AEEC#s 02084, 02085, 05097, 05107, 06199, 0810912) have shown this to be observably painless and causing minimal observable discomfort to the animal. However, in the event the animals show signs of distress (animals will be monitored daily using attached monitoring and intervention checklists) such as loss of body weight (more than 10% over a 24 h period), lethargy, piloerection, hunched appearance, loss of appetite and loss of grooming, they will be immediately killed by an overdose (intraperitoneal injection) of anaesthetic (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg). A 1 ml syringe with a 27 gauge needle will be used for this injection. Death will be ensured by opening of the chest cavity. Each experiment will have a matched (exposure period) sham smoked group of animals that will be placed in the perspex chamber but not exposed to cigarette smoke. Similar experiments will also be performed in genetically modified mice to determine the importance of defects of certain genes in the development of COPD.

Cigarette smoke exposure (6 month protocol)

Most of the smoking protocols will involve the 4 day protocol, which produces many features of COPD, but for some purposes we use longer term models (6 months, 5 days per week) so that the full COPD symptom profile is observed in the mice, including a decrease in muscle weight and damage to alveoli ie emphysema (see below). For this model we propose to expose mice to cigarette smoke in the same fashion as the 4 day smoke protocol, however rather than exposing mice to 9 cigarettes per day we propose 6 cigarettes per day as follows: It takes approximately 3 min to smoke 1 cigarette using a constant rate of 10 sec to draw up the syringe barrel. The box lid is left on for another 12 min and then removed for 5 min of fresh air. This is completed twice in succession 3 times per day, ie the maximum smoke exposure is 6 cigarettes per day over at least an 8 hour period. We and others have shown that long-term smoking of mice is required to see damage to alveoli (the small sacs in the lung where gas exchange takes place) which is the other pathological feature of COPD. Data from long-term smoking protocols (6 cigarettes/day, 5d/week for 4 weeks or 6 months) show that mice do not lose weight but gain less weight than no-smoke control mice. Previous experiments (AEEC #02085, 0810912) have shown this to be painless and non-noxious and there is minimal discomfort to the animal. However, in the event the animals shows signs of distress (animals will be monitored daily using attached monitoring and intervention checklists) such as loss of body weight (more than 10% over a 24 h period), lethargy, piloerection, hunched appearance, loss of appetite and loss of grooming, they will be immediately killed by an overdose (intraperitoneal injection) of anaesthetic (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg). A 1 ml syringe with a 27 gauge needle will be used for this injection. Death will be ensured by opening of the chest cavity. Each experiment will have a matched (exposure period) sham smoked group of animals that will be placed in the Perspex chamber but not exposed to cigarette smoke. Similar experiments will also be performed in genetically modified mice to determine the importance of defects of certain genes in the development of COPD.

Cigarette smoke exposure and virus infection protocol

In some experiments, after exposure to 4 days of cigarette smoke exposure (same protocol as above), the mouse-adapted influenza A strains Mem71 (H3N1 - low pathogenicity) and HKx31 (H3N2 - intermediate pathogenicity) will be used to exacerbate the underlying smoke-induced inflammation. Mice are placed in a ~0.5L plastic tub where around 1mL of methoxyflurane is put on a wad of cotton wool and then 5 layers of gauze are placed on top, so that the mice does not directly contact the anesthetic (mild irritant). The mouse is then put into the tub and a timer started, it takes around 2 min for anesthesia to develop, as assessed by loss of the "righting reflex" when the tub is gently rocked. The mouse is then infected with Influenza by gently pipetting 50 µl of Influenza (Mem 71 dose is 31,000 plaque forming units diluted in cell culture medium; HKx31 is 1x10^6 plaque forming units diluted in cell culture medium) onto the nose. Infection of influenza is allowed to replicate within the animal before peak numbers occur approximately 3-4 days after infection. Such influenza infection in normal mice causes mild to moderate airway inflammation but no overt disease. We have done influenza infection of mice that were smoke exposed and while inflammation was increased in comparison to smoke alone or virus alone mice, there was no apparent increase in observable illness (AEC# 02084, 05107, 0810912). Genetically modified mice will not be used in these experiments.

Animal monitoring

Mice will be monitored daily for signs of illness such as loss of body weight (more than 10% in a 24 hour period), lethargy,
ruffled fur, hunched appearance, panting, loss of appetite (as seen by monitoring food intake), isolation from the group and loss of grooming (ie criteria set out in attached monitoring and intervention checklists). Weights will be recorded daily in all experiments except for the 6 month smoke studies in which weights will be recorded on Monday, Wednesday, Friday. Mice will be observed daily during the experimental protocol to ensure that no overt lung responses, respiratory distress or discomfort are elicited by LPS treatment, cigarette smoke exposure to virus infection. If an adversely severe reaction occurred the mice would show reduced mobility, panting and piloerection - in such circumstances they would be immediately killed by anesthetic overdose (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg). Death will be ensured by opening of the chest cavity.

Euthanasia of mice

At the end of the experimental protocol, mice will be killed by an intraperitoneal anaesthetic overdose (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg) administered via a 27 gauge needle and subjected to the procedures described below. Death will be ensured by opening of the chest cavity.

Procedures after the animals have been killed:

1. Blood samples will be taken from the inferior vena cava of each animal and analysed to determine signs of cellular inflammation.

2. The diaphragm is opened, lungs lavaged (method to obtain fluid from the lungs to determine level of inflammation) with PBS (400 µl, followed by 3 times 300 µl aliquots) via the trachea.

3. The chest cavity is opened and lungs removed and examined for inflammatory changes using (i) Cellular inflammation (principally lavage); (ii) RNA extraction for gene profiling using gene microarrays/QPCR and (iii) histology and immunohistochemistry. These procedures cannot be concurrently performed in the same animal and the experiments have been designed to extract the maximum information from as few mice as possible.

4. Organs such as the liver, kidney, fat pads, muscles, heart, spleen, brain and ilium will be dissected and weight to examine non-specific effects of test compounds.

8. PERSONNEL

<table>
<thead>
<tr>
<th>Name</th>
<th>Vlahos, A/Prof Ross</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>PhD, University of Melbourne, Bachelors Degree (Honours), University of Melbourne</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
</tr>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
</tr>
<tr>
<td>Mouse</td>
<td>Oral gavage</td>
</tr>
<tr>
<td>Mouse</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intravenous administration of agents</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
</tr>
</tbody>
</table>
### OTHER INVESTIGATOR

<table>
<thead>
<tr>
<th>Name</th>
<th>Seow, Mr Huei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelors Degree (Honours), University of Melbourne, Bachelors Degree, University of Melbourne</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Bozinovski, A/Prof Steven</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>PhD, University of Melbourne, Bachelors Degree (Honours), La Trobe University</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Anthony, Dr Desiree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelors Degree (Honours), Deakin University, PhD, University of Melbourne</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
<td>Yes</td>
<td>Dr Ross Vlahos</td>
<td>More than 20</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Donovan, Chantal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Ms Chantal Donovan completed her BSc (Hons) in the Department of Pharmacology in 2011. She is now in the second year of her PhD in Dr Jane Bourke's laboratory. We now wish to collaborate</td>
</tr>
</tbody>
</table>

Page - 12 - Date: 09 July 2019 09:07:57
to examine the effects of cigarette smoke exposure on mouse large and small airways contractility.

<table>
<thead>
<tr>
<th>Department</th>
<th>Pharmacology And Therapeutics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td></td>
</tr>
<tr>
<td>Technique /Procedure</td>
<td></td>
</tr>
<tr>
<td>Times Performed</td>
<td></td>
</tr>
<tr>
<td>Training Required</td>
<td></td>
</tr>
<tr>
<td>Training Provided by</td>
<td></td>
</tr>
<tr>
<td>Expertise of Trainer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Intranasal administration of agents</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal injection of agents</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Yatmaz, Mr Selcuk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelor of Pharmacy (Honours), University of Melbourne, Bachelors Degree, University of Melbourne</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
<tr>
<td>Species</td>
<td></td>
</tr>
<tr>
<td>Technique /Procedure</td>
<td></td>
</tr>
<tr>
<td>Times Performed</td>
<td></td>
</tr>
<tr>
<td>Training Required</td>
<td></td>
</tr>
<tr>
<td>Training Provided by</td>
<td></td>
</tr>
<tr>
<td>Expertise of Trainer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Intranasal administration of agents</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Oral gavage</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal injection of agents</td>
<td>more than 20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Oostwoud, Ms Leanne</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelor of Pharmacy (University of Groningen, Netherlands)</td>
</tr>
<tr>
<td>Leanne will now come to my lab for 6 months (2 December 2013 to 2 July 2014) to conduct her master of research project as part of her studies.</td>
<td></td>
</tr>
<tr>
<td>Department</td>
<td>Organisation Name not found</td>
</tr>
<tr>
<td>Species</td>
<td></td>
</tr>
<tr>
<td>Technique /Procedure</td>
<td></td>
</tr>
<tr>
<td>Times Performed</td>
<td></td>
</tr>
<tr>
<td>Training Required</td>
<td></td>
</tr>
<tr>
<td>Training Provided by</td>
<td></td>
</tr>
<tr>
<td>Expertise of Trainer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Intranasal administration of agents</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Oral gavage</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose</td>
<td>0</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
</tr>
</tbody>
</table>

**Name**: Hansen, Dr Michelle  
**Qualifications**: PhD, University of Melbourne  
**Department**: Pharmacology And Therapeutics

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Name**: Passey, Dr Samantha  
**Qualifications**: PhD, University of Bristol, Bachelors Degree, University of Bristol  
**Department**: Pharmacology And Therapeutics

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Oral gavage</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Name**: Khau, Dr Thippadey  
**Qualifications**: PhD, University of Melbourne, Bachelors Degree (Honours), University of Melbourne, Bachelors Degree, University of Melbourne  
**Department**: Pharmacology And Therapeutics

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Oral gavage</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
<td>Times Performed</td>
<td>Training Required</td>
<td>Training Provided by</td>
<td>Expertise of Trainer</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Austin, Victoria Margaret Blaire</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelor of Science</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Oral gavage</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Bernardo, Ivan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelor of Science</td>
</tr>
<tr>
<td>Department</td>
<td>Pharmacology And Therapeutics</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Oral gavage</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Ngo, Mr Hoai Buu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Bachelor of Science</td>
</tr>
<tr>
<td>Department</td>
<td>Organisation Name not found</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Expose mice to cigarette smoke</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intranasal administration of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Intraperitoneal injection of agents</td>
<td>0</td>
<td>Yes</td>
<td>Mr Huei Jiunn Seow</td>
<td>More than 20 times</td>
</tr>
</tbody>
</table>
9. MONITORING

9.1 Day-to-day monitoring during the project.

Who will monitor the animals on weekdays?

A/Prof Ross Vlahos, Mr Huei Seow

Who will monitor the animals after hours (incl. Weekends and holidays)?

A/Prof Ross Vlahos, Mr Huei Seow

9.2 Day-to-day monitoring during the project: What specific signs will be monitored and how frequently?

Attach a copy of the monitoring checklist you will use to record these observations.

Once the animals arrive from the source (e.g., ARC) the experimental procedures will begin after one week as animals require sufficient time to acclimatise to their new environment. During this acclimatisation period, animals are monitored twice by the investigators and daily by the Biological Research Facility staff. As animals have not undergone any experimental procedures at this time there should be no signs of distress; however, we will monitor to determine if animals are fighting, and whether each animal looks to be in good condition.

9.3 Monitoring during and after procedures/interventions: What specific signs will be monitored and how frequently?

Attach a copy of the monitoring checklist you will use to record these observations.

During the procedures animals will be monitored daily for overt signs of distress including piloerection, decreased interaction with peers, loss of appetite (assessed by changes in body weight) using the attached monitoring checklist. In addition, body weight will be monitored every Monday, Wednesday, and Friday in the 6 month studies.

9.4 What clinical, behavioural or other signs will be used to indicate that intervention is needed to alleviate an animal’s pain or suffering? What action will be taken if these indicators are reached?

Signs of distress are detailed in the attached intervention checklist and include including piloerection, decreased interaction with peers, loss of appetite (assessed by changes in body weight). Determination of whether an animal will be euthanized based on clinical and behavioral signs will be made by an experienced investigator and will depend on the severity of the symptom. Mice who display 1 or more of the “severe” signs on the attached Intervention Criteria Checklist or have any obvious signs of pain will be euthanized immediately with an overdose of anesthetic (a mixture of ketamine 240 mg/kg and xylazine 40 mg/kg) administered via a 27 gauge needle. Death will also be ensured by opening of the chest cavity.

9.5 Who is responsible for the management of emergencies?

A/Prof Ross Vlahos, Mr Huei Seow

10. FATE OF ANIMALS
10.1 What will be the maximum period of time that an individual animal or group of animals will be used in this project?

Majority of animals will be kept for up to 2 weeks. However, in long-term cigarette smoke exposure protocols, mice may be kept for up to 7 months.

10.2 What will happen to the animals at the completion of the project?

Animals will be brought down to our laboratory [N804, Level 8 of the Medical Building, North Wing] in transport boxes via the service lift. Animals will then be killed by an anesthetic overdose [0.35 ml intraperitoneal, a mixture of ketamine [240 mg/kg] and xylazine [40 mg/kg], blood taken via the inferior vena cava, the diaphragm opened and the lungs lavaged and removed for further analysis.

10.3 If the animals are to be killed, how will this be done and by whom? Include information about agents, dose rates, method and route of administration and experience of personnel.

Animals are to be killed by an anesthetic overdose [0.35 ml], a mixture of ketamine [240 mg/kg] and xylazine [40 mg/kg] administered into the intraperitoneal cavity using a 27 gauge syringe. Only an experienced investigator will be allowed to administer this agent to the animal. Each of the investigators listed on this ethics application has an experience level above 20 times. Death will also be ensured by opening of the chest cavity.

10.4 What will be the method of disposal of dead animals?

Animals will be placed in black plastic bags and left in the biological hazard freezer in the BRF on the 9th floor. The bodies will then be disposed of by the BRF [incinerated by an approved company].

11. ADDITIONAL DETAILS

11.1 Risk Management

11.1.1 Does the research involve procedures or agents that might pose a health risk to other animals and/or personnel? If Yes, please explain the risk and describe what precautions will be taken.

Yes - We have developed a mouse model of influenza-induced COPD exacerbation, by infecting mice with the mouse-adapted Influenza A virus strains Mem71 [H3N1] and HKx31 [H3N2]. These viruses are of low [Mem71] and intermediate [HKx31] virulence. Mice will be lightly anesthetized by the inhalation of methoxyflurane by placing them in a small plastic tub [about 0.5 litre] where around 1 ml of methoxyflurane is placed on a wad of cotton wool and then 5 layers of gauze are placed on top to prevent direct contact with the anesthetic. The mouse is put into the tub and a timer started, it takes around 2 minutes for anesthesia to develop, as assessed by loss of the - righting reflex - when the tub is gently rocked. Anesthetizing the animals allows the experimenter to treat the animal quickly and effectively as they are immobilized and as mice are obligate nose breathers the saline containing influenza virus will be distributed uniformly throughout the lung. Animals are then placed in a recovery box for approximately 5 minutes and make a full recovery. The influenza viruses are not lethal at these doses of infection. These viruses, whilst from human origin, has been extensively laboratory adapted so that they are no longer pathogenic to humans. Although it is well established that these mouse-adapted Influenza A virus strain is not contagious, mice will be housed in sterile micro-isolator cages in order to protect our mice from infection and to further reduce the very remote risk of infection to other animals in the BRF. In addition, the procedure will be performed in a safety cabinet and the person performing the procedure will wear a mask/hood which has fresh air pumped through it to protect the investigator from breathing in methoxyflurane.

11.1.2 Is the acquisition, holding, or use of the animals subject to any permit, law or regulation of the State or Commonwealth (e.g., OGTR, protected native or imported)? If yes, please specify permit numbers.
11.2 Glossary of Scientific Terms

<table>
<thead>
<tr>
<th>Scientific Term</th>
<th>Lay Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Obstructive Pulmonary Disease (COPD)</td>
<td>A disease also known as emphysema in which there is chronic lung inflammation and damage resulting in shortness of breath. People with emphysema rely on oxygen therapy in the final years of their life. There is no cure nor effective treatment for this disease.</td>
</tr>
<tr>
<td>Acute Exacerbations of Chronic Obstructive Pulmonary Disease (AECOPD)</td>
<td>People with Chronic Obstructive Pulmonary Disease often experience rapid declines in health often caused by lung infections. The rapid decline in lung function consists of high levels of lung inflammation, extreme shortness of breath and sometimes death.</td>
</tr>
<tr>
<td>Reactive Oxygen Species (ROS)</td>
<td>Substances released from cells of the lung and white blood cells which cause lung inflammation and lung damage.</td>
</tr>
<tr>
<td>NADPH oxidase-1 (NOX-1)</td>
<td>An enzyme responsible for the generation of Reactive Oxygen Species</td>
</tr>
<tr>
<td>NADPH oxidase-2 (NOX-2)</td>
<td>An enzyme responsible for the generation of Reactive Oxygen Species</td>
</tr>
<tr>
<td>NADPH oxidase-4 (NOX-4)</td>
<td>An enzyme responsible for the generation of Reactive Oxygen Species</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>A bacterial wall component which causes Acute Exacerbations of Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>Gpx-1</td>
<td>An enzyme that converts the toxic hydrogen peroxide to non-toxic water and oxygen</td>
</tr>
<tr>
<td>IL-17</td>
<td>A cytokine produced by white blood cells within the body</td>
</tr>
<tr>
<td>uPA</td>
<td>A protein produced by white blood cells which breaks down blood clots</td>
</tr>
<tr>
<td>IL-23</td>
<td>A cytokine present in white blood cells within the body</td>
</tr>
<tr>
<td>tPA</td>
<td>A protein present in white blood cells which breaks down blood clots</td>
</tr>
<tr>
<td>Cytokine</td>
<td>Protein produced and released by individual cells for the purpose of transmitting distinct messages to the cell</td>
</tr>
</tbody>
</table>

11.3 Attachments

The following attachments should accompany this application:

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Attached Via Themis</th>
<th>Hard Copy Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miscellaneous</td>
<td>Cover letter addressing requested changes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Monitoring checklist proforma</td>
<td>Monitoring Checklist</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Intervention Criteria</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Animals Requested</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

11.4 Funding and Contracts

11.4.1 Identify the principal source of funding for this project (internal/external/commercial or private).

External funding agency

11.4.2 Is this project Commercial in Confidence?
11.4.3 Is this project covered by a research contract?
Yes

12. GENETIC MODIFICATION OR CLONING OF ANIMALS

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>NOX-2 deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Genotype (if applicable for cloned animals):

12.1.1 Describe the function/s of the gene/s that have been/will be modified.

The NOX-2 gene encodes for a protein that is highly expressed in professional phagocytes such as neutrophils and macrophages that generate large amounts of superoxide as part of their armoury of bactericidal mechanisms. Although NOX-2 containing oxidases intentionally generate Reactive Oxygen Species for functions related to innate immunity and signal transduction, we believe that NOX-2 is up-regulated in COPD and other lung inflammatory conditions, which is responsible for generating unusually higher levels of Reactive Oxygen Species that ultimately damage the lung.

12.1.2 Explain the relevance of the modification to the project.

Our microarray and proteomic studies have shown that a number of oxidative stress molecules are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease. We believe that NADPH oxidase (NOX), the enzyme responsible for generation of lung destroying Reactive Oxygen Species (ROS), may be a novel therapeutic target for COPD and Acute Exacerbations of COPD, as it is upstream of other anti-oxidants (eg superoxide dismutase). The NOX-2 gene gene encodes for a protein that is highly expressed in cells of the lung and blood (neutrophils and macrophages). We propose that mice deficient in NOX-2 may be protected from cigarette smoke and LPS-induced lung injury because these mice are incapable of producing lung destroying Reactive Oxygen Species in response to LPS and cigarette smoke. Thus, we now wish to explore whether animals deficient in NOX-2 are protected from cigarette smoke and LPS-induced lung inflammation.

12.1.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.1.4 What will be the fate of animals that are not of the appropriate genotype?

NOX-2 deficient mice will be sent to us by our collaborator Dr Stavros Selemidis. The genotyping of all animals sent to us is done by researchers at Monash University before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:

12.1.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been characterized. The animal has no behavioral, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.
12.1.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.1.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>IL-17 deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Genotype (if applicable for cloned animals):

12.2.1 Describe the function/s of the gene/s that have been/will be modified.

Interleukin-17 (IL-17) is a cytokine produced at local sites (in particular immune cells called T cells) within the body.

12.2.2 Explain the relevance of the modification to the project.

The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of IL-17 in the effect of COPD and AECOPD.

12.2.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.2.4 What will be the fate of animals that are not of the appropriate genotype?

IL-17 deficient mice will be sent to us by our collaborator A/Prof Brendan Jenkins. The genotyping of all animals sent to us is done by researchers at Monash Institute for Medical Research before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:

12.2.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been characterised. The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan. IL-17 deficient mice have a normal physical appearance and breed normally. These mice only display impaired immune responses if subjected to certain experimental challenges (eg lung asthma models) (Nakae et al. 2002. Antigen-Specific T Cell Sensitization Is Impaired in IL-17-Deficient Mice, Causing Suppression of Allergic Cellular and Humoral Responses Immunity 17: 375-387).

12.2.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.
Breeding (completion of this section is required only if animals are bred in-house):

12.2.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

12.3

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>NOX-1 deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Genotype (if applicable for cloned animals):

12.3.1 Describe the function/s of the gene/s that have been/will be modified.

The NADPH oxidase (NOX)-1 gene encodes for a protein that is highly expressed in epithelial cells in the lung and other tissues such as the colon and vasculature. NOX-1 is the major component of an enzyme complex that generates toxic Reactive Oxygen Species (such as superoxide) in response to invading pathogens and cigarette smoke. We believe that NOX-1 is up-regulated in COPD and other lung inflammatory conditions, which is responsible for generating Reactive Oxygen Species that damage the lung.

12.3.2 Explain the relevance of the modification to the project.

Our microarray and proteomic studies have shown that a number of oxidative stress molecules are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease. We believe that NOX, the enzyme responsible for generation of lung destroying Reactive Oxygen Species (ROS), may be a novel therapeutic target for COPD and Acute Exacerbations of COPD, as it is upstream of other anti-oxidants (eg superoxide dismutase). The NOX-1 gene encodes for a protein that is highly expressed in epithelial cells in the lung. We propose that mice deficient in NOX-1 may be protected from cigarette smoke and LPS-induced lung injury because these mice are incapable of producing lung destroying Reactive Oxygen Species in response to LPS and cigarette smoke. Thus, we now wish to explore whether animals deficient in NOX-1 are protected from cigarette smoke and LPS-induced lung inflammation. In all of our proposed experiments we will use mice at 6-8 weeks of age, an age where baseline phenotypes would unlikely severely impact on the well-being of the animals.

12.3.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.3.4 What will be the fate of animals that are not of the appropriate genotype?

NOX-1 deficient mice will be sent to us by our collaborator Dr Stavros Selemidis. The genotyping of all animals sent to us is done by researchers at Monash University before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:

12.3.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.
Yes - The phenotype of this strain of animal has been characterised. The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.

12.3.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.3.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

12.4 Type/Species | Breed/Strain | Background Strain
--- | --- | ---
Mouse (Genetically Modified) | NOX-4 deficient | C57BL/6

Genotype (if applicable for cloned animals):

12.4.1 Describe the function/s of the gene/s that have been/will be modified.

The NOX-4 gene encodes for a protein that is highly expressed in epithelial and endothelial cells in the lung. NOX-4 is homologous to NOX-1 and NOX-2 and is an important component of an enzyme complex that generates toxic Reactive Oxygen Species such as hydrogen peroxide in those cells. Whilst NOX-4 expression is generally low, we believe it is up-regulated in COPD and other lung inflammatory conditions and responsible for generating reactive oxygen species that damage the lung.

12.4.2 Explain the relevance of the modification to the project.

Our microarray and proteomic studies have shown that a number of oxidative stress molecules are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease. We now believe that NADPH oxidase (NOX), the enzyme responsible for generation of lung destroying Reactive Oxygen Species (ROS), may be a novel therapeutic target for COPD and Acute Exacerbations of COPD, as it is upstream of other anti-oxidants (e.g., superoxide dismutase). The NOX-4 gene encodes for a protein that is highly expressed in epithelial and endothelial cells in the lung. We propose that mice deficient in NOX-4 may be protected from cigarette smoke and LPS-induced lung injury because these mice are incapable of producing lung destroying Reactive Oxygen Species in response to LPS and cigarette smoke. Thus, we now wish to explore whether animals deficient in NOX-4 are protected from cigarette smoke and LPS-induced lung inflammation.

12.4.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.4.4 What will be the fate of animals that are not of the appropriate genotype?

NOX-4 deficient mice will be sent to us by our collaborator Dr Stavros Selemidis. The genotyping of all animals sent to us is done by researchers at Monash University before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:

12.4.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the
modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been characterised. The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.

12.4.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.4.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

12.5

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>MyD88 deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Genotype (if applicable for cloned animals):

12.5.1 Describe the function/s of the gene/s that have been/will be modified.

MyD88 is an adaptor protein involved in the control of innate immunity. The Myd88 gene encodes for a protein that is highly expressed in various organs and cell types such as the lung and macrophages. MyD88 is a major component of the TLR receptor complex that coordinates the host's defence responses to invading pathogens and other noxious airborne insults. We believe that MyD88 controls inflammatory processes in COPD and other lung inflammatory conditions, which contribute to lung tissue damage.

12.5.2 Explain the relevance of the modification to the project.

The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of MyD88 in the pathology or protection of COPD. Our preliminary studies have shown that a number of inflammatory molecules are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease, and that these events are associated with activation of the master transcription factor Nuclear Factor kappa B (NFkB). However, it is not clear how NFkB-mediated inflammation in COPD and Acute Exacerbations of COPD (AECOPD) typically triggered by infectious pathogens are controlled. The TLR receptor pathway is primarily responsible for recognition of various airborne respiratory pathogens and other endogenous stress ligands that initiate the early host defence response. There are at least 11 TLR family members that utilise MyD88 as an adaptor molecule to initiate intracellular signalling cascades responsible for induction of various inflammatory mediators. Since MyD88 is a common and central adaptor for all TLR family members with the exception of TLR3, MyD88 may be an alternative (and perhaps better) therapeutic target for COPD and AECOPD. Particularly, the role of TLRs in COPD and AECOPD is currently not well defined and the use of this single genetic modification will provide insight into the entire TLR family. By selectively targeting the MyD88 gene we can elucidate whether TLR signalling contributes to the inflammatory mechanisms driving COPD and whether targeting MyD88 would be a better therapeutic strategy. We propose that mice deficient in MyD88 may be protected from cigarette smoke and LPS-induced lung injury because these mice are incapable of producing lung destroying inflammatory mediators such as cytokines, chemokines and reactive oxygen species in response to LPS and cigarette smoke. Thus, we now wish to explore whether animals deficient in MyD88 are protected from cigarette smoke and LPS-induced lung inflammation.

12.5.3 Will tissue be collected to use for genotyping? If yes, describe how and when.
12.5.4 What will be the fate of animals that are not of the appropriate genotype?

MyD88 deficient mice will be given to us by our collaborator Dr Peter Crack. The genotyping of all animals given to us is done by researchers at The University of Melbourne before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:

12.5.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been well characterised. The MyD88 knockout mice demonstrate increased apoptosis after lung injury. They also display abnormal immune physiology, decreased inflammatory response, abnormal response to infection and abnormal cytokine secretion. The animal has no reproductive and developmental features that affect its health, welfare, breeding or lifespan.

12.5.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.5.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

12.6 Type/Species | Breed/Strain | Background Strain
---|---|---
Mouse (Genetically Modified) | IL-23 deficient | C57BL/6

Genotype (if applicable for cloned animals):

12.6.1 Describe the function/s of the gene/s that have been/will be modified.

Interleukin-23 is a cytokine produced by immune cells within the body.

12.6.2 Explain the relevance of the modification to the project.

The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of IL-23 in the effect of COPD and AECOPD.

12.6.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.6.4 What will be the fate of animals that are not of the appropriate genotype?
IL-23 deficient mice will be sent to us by our collaborator A/Prof Matthias Ernst. The genotyping of all animals sent to us is done by researchers at Ludwig Institute for Cancer Research before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

**Phenotype:**

12.6.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been characterised. The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.

12.6.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

**Breeding (completion of this section is required only if animals are bred in-house):**

12.6.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>Gpx-1 deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

**Genotype (if applicable for cloned animals):**

12.7.1 Describe the function/s of the gene/s that have been/will be modified.

Glutathione peroxidase-1 (Gpx-1) is an endogenous anti-oxidant enzyme that catalyses the potentially toxic hydrogen peroxide to water and oxygen. The mice that lack Gpx-1 are under oxidative stress owing to a reduced capacity to clear hydrogen peroxide.

12.7.2 Explain the relevance of the modification to the project.

The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of Gpx-1 in the effect of COPD and AECOPD.

12.7.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.7.4 What will be the fate of animals that are not of the appropriate genotype?

Gpx-1 deficient mice will be given to us by our collaborator Dr Peter Crack. The genotyping of all animals sent to us is done by researchers at The University of Melbourne before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.
Phenotype:

12.7.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The mice that lack Gpx-1 are under oxidative stress owing to a reduced capacity to clear hydrogen peroxide. It has been reported Gpx-1 deficient mice demonstrated increased inflammation and cell death following a cold-induced brain injury or cerebral ischemia. At 8 months of age Gpx-1 deficient mice weigh 20 % less than wild-type controls but no differences were detected in liver, kidney or heart tissue weights compared to wild-type controls. Gpx-1 deficient mice have no known histopathological or developmental abnormality - they are healthy and fertile (longest reported study up to 15 months of age) consistent with a limited role for gpx-1 during normal development and under physiological conditions (Ho et al, 1997; Cheng et al 1997; de Haan et al 1998).

12.7.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.7.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

12.8

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>tPA deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Genotype (if applicable for cloned animals):

12.8.1 Describe the function/s of the gene/s that have been/will be modified.

Tissue type-plasminogen activator (t-PA) is involved in fibrin breakdown. Fibrin is formed as a clot at the end of the coagulation pathway.

12.8.2 Explain the relevance of the modification to the project.

The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of tPA in the effect of COPD and AECOPD.

12.8.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.8.4 What will be the fate of animals that are not of the appropriate genotype?

tPA deficient mice will be sent to us by our collaborator Prof John Hamilton. The genotyping of all animals sent to us is done by researchers at The University of Melbourne (Department of Medicine) before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:
12.8.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been characterised. The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.

12.8.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.8.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

12.9

<table>
<thead>
<tr>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Background Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Genetically Modified)</td>
<td>uPA deficient</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

Genotype (if applicable for cloned animals):

12.9.1 Describe the function/s of the gene/s that have been/will be modified.

Urokinase-plasminogen activator (u-PA) is produced by macrophages and is involved in breakdown of fibrin and cell migration. Fibrin is formed as a clot at the end of the coagulation pathway.

12.9.2 Explain the relevance of the modification to the project.

The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of uPA in the effect of COPD and AECOPD.

12.9.3 Will tissue be collected to use for genotyping? If yes, describe how and when.

No

12.9.4 What will be the fate of animals that are not of the appropriate genotype?

uPA deficient mice will be sent to us by our collaborator Prof John Hamilton. The genotyping of all animals sent to us is done by researchers at The University of Melbourne (Department of Medicine) before we receive them. Thus, the genotype of all animals sent to us is already known by the time we receive them.

Phenotype:

12.9.5 Is the phenotype of this strain well characterized? If yes, briefly describe the known behavioural, physiological, reproductive and developmental features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals. If no, briefly describe the potential or anticipated behavioural, physiological, reproductive and developmental
features of the strain and identify whether the modification/s will affect the health, welfare, breeding or lifespan of the animals.

Yes - The phenotype of this strain of animal has been characterised. The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.

12.9.6 Does the strain require special husbandry? If yes, describe the particular requirements for care.

No

Breeding (completion of this section is required only if animals are bred in-house):

12.9.7 Describe, or attach Standard Operating Procedures that describe the breeding program (e.g., heterozygous, homozygous, back-crossing) that will be used to produce the genetically modified or cloned animals to be used in the project.

13. AUTHORISATIONS

The Research Office has verified that the following nominated personnel have signed off on this project application online through THEMIS on the given date/s:

<table>
<thead>
<tr>
<th>Role</th>
<th>Name</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Supervisor</td>
<td>Vlahos, A/Prof Ross</td>
<td>06 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Hansen, Dr Michelle</td>
<td>07 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Bozinovski, A/Prof Steven</td>
<td>07 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Anthony, Dr Desiree</td>
<td>12 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Seow, Mr Huei</td>
<td>06 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Khau, Dr Thippadey</td>
<td>06 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Donovan, Chantal</td>
<td>06 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Yatmaz, Mr Selcuk</td>
<td>07 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Passey, Dr Samantha</td>
<td>11 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Austin, Victoria Margaret Blaire</td>
<td>12 March 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Bernardo, Ivan</td>
<td>12 March 2014</td>
</tr>
<tr>
<td>Animal Facility Manager</td>
<td>Kesar, Ms Marica</td>
<td>12 March 2014</td>
</tr>
</tbody>
</table>

The Project Supervisor has acknowledged that all trainers listed on this application have the relevant expertise and that they have accepted responsibility to train their nominated researcher/s to be competent in the necessary procedures.
Title: Identification of novel therapeutics for Chronic Obstructive Pulmonary Disease  
Ethics ID: 1112185

On behalf of the Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee, the Chair, in consultation with the Acting Animal Welfare Office, has approved the request for amendment relating to add new investigators Dr Michelle Hansen, Dr Samantha Passey, Dr Thippadey Khau, Ms Victoria Austin, Mr Ivan Bernardo and Mr Hoai Buu (Bruce) Ngo to the above, currently approved project.

You may treat this email as official notification of approval to proceed with the project as amended.

Regards,
Dr Lei Shong Lau
Animal Ethics Officer
Office for Research Ethics and Integrity

Phone: 
Email:
8 December 2015

Associate Professor Ross Vlahos  
School of Health Sciences  
RMIT University

Dear Ross,

AEC 1521: Use of genetically deficient and wild-type mice for the identification of novel therapeutics for Chronic Obstructive Pulmonary Disease.

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from 8 December 2015 until 8 December 2018. An approved version of the application is attached.

Animals
Your application has been approved to use up to n=1848 mice (BALB/c), n=216 mice (C57BL/6 GPX-1 knockout or wild-type), n=216 mice (C57BL/6 NOX-1 knockout or wild-type), n=216 mice (C57BL/6 NOX-2 knockout or wild-type), n=216 mice (C57BL/6 NOX-4 knockout or wild-type), and n=216 mice (C57BL/6 IL-17 knockout or wild-type) over the duration of the project.

Please note that no genetically modified animals may be ordered, or used in this project unless appropriate approvals have been obtained from an Institutional Biosafety Committee.

The use of animals in scientific procedures is strictly regulated by the Australian code of practice for the care and use of animals for scientific purposes. The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

Responsibilities of investigators
1. Associate Professor Ross Vlahos
2. Associate Professor Steven Bozinovski
3. Dr Samantha Louise Passey
4. Mr Huei Jiunn Seow
5. Mr Ivan Bernardo
6. Mr Selcuk Yatmaz
7. Ms Victoria Austin
8. Ms Tricia Murphy
9. Dr Joo Lee Cham
10. Ms Emma Colinson
11. Ms Vikki Smyth

Responsibilities of investigators are described in the Australian code of practice for the care and use of animals for scientific purposes (section 3). Investigators have a ‘personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project’ (s.3.1.1).
Amendments and extensions
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with ‘minor’ amendment requests. Major amendments to projects normally require a new project application.

Adverse events or unexpected outcomes
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.

Unwell animals must be immediately reported via the care forms available at the RMIT Animal Facility. In the case of any emergency, the Animal Welfare Officer, Dr Rebbecca Wilcox, may be contacted on 0409 521 234 at any time. In case of any unexpected animal death, the researcher has a responsibility to organise an autopsy so as to determine the cause of death.

Investigator guidelines for record keeping
Investigators are required to adhere to the strict guidelines regarding record keeping for their project. Note that records associated with a project ‘should be available for audit by the institution and authorised external reviewers’. Failure to maintain proper records may result in a compliance breach of the Code and place at risk the researcher’s capacity to carry out research with animals.

Conditions of approval
The AEC may apply conditions of approval beyond the submission of annual/final reports. Please note that no transgenic animals may be ordered, or used in this project unless appropriate approvals have been obtained from an Institutional Biosafety Committee.

Reports
Approval to continue a project is conditional on the submission of annual and final reports. Annual reports are requested in December each year, and must be submitted whether or not the project has commenced or is inactive. Report forms are available at www1.rmit.edu.au/staff/research/research-integrity-and-governance/animal-ethics.

Failure to submit reports will mean that a project is no longer approved, and/or that approval will be withheld from future projects.

All reports or communication regarding this project are to be forwarded to the research ethics coordinator at animalethics@rmit.edu.au

On behalf of the AEC I wish you well with your research.

Dr Brad Hayward
Research Ethics Coordinator
On behalf of
RMIT Animal Ethics Committee

cc:  Ms Tricia Murphy, RAF manager
KEY RESPONSIBILITIES

All scientific procedures using animals must be carried out in accordance with the Prevention of Cruelty to Animals Act 1986 Act (the Act), associated Regulations and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (the Code).

These legislative requirements specify that an Animal Ethics Committee (AEC) must verify that the use of animals for research or teaching is justified and adheres to the principles of Replacement, Reduction and Refinement. All proposed animal use must be approved by an AEC before commencing the project.

Before completing this application form investigators should be familiar with the following as applicable:

· The Australian Code of Practice for the Care and use of Animals for Scientific Purposes (the Code).
· The Code of Practice for the Housing and Care of Laboratory mice, rats, guinea pigs and rabbits (the Housing Code)
· Part III of the Prevention of Cruelty to Animals Act 1986 and Regulations 2008 (the Act)

Knowledge of these legal requirements will assist you in completing this application in a satisfactory manner. The above documents can be found at www.dpi.vic.gov.au/animalwelfare/

NOTES ON THE COMPLETION OF THIS APPLICATION FORM

Completion Instructions

This is a dynamic form designed to be completed and submitted electronically. Please do not print this form. Save a blank copy to your computer and open it with the free Adobe Reader software (version 9.0 or later). If you do not have Adobe Reader installed, or are using an older version, you can download the latest free version for Mac and PC from the Adobe website. Mac Users please note that the default program 'Preview' will not display this form correctly - you will need to install Adobe Reader to view and edit this form.

Insert your answers in the boxes provided below each question. When necessary boxes will expand to accommodate the length of your answer. A response is required for each question. Write "Not applicable", if necessary.

Applications must be written in plain English. It should be assumed that assessors have either no scientific knowledge or no knowledge of your area of research. Where scientific language is unavoidable, it must be supported by a suitable lay description or a glossary of terms. It is not appropriate to include sections from grant applications containing excessive detail of procedures unrelated to the use of animals. It is highly recommended that you ask a colleague and a person with a non-scientific background to read the application before it is submitted.

Where SOPs are available for a procedure, please refer to the SOP number and title rather than describe the procedure again.

Submission Instructions

Complete the form electronically, save it and attach it to an email addressed to the Secretary of the Animal Ethics Committee at animalethics@rmit.edu.au. Submission of applications is required by the agenda deadline for each month's Animal Ethics Committee meeting. Please see the animal ethics website or contact the Ethics Officer for meeting and submission deadline dates.

To optimise and expedite the application process it is recommended that you consult with the Animal Welfare Officer on design of projects and completion of application forms. The Animal Welfare Officer can be contacted on: awo@rmit.edu.au or 9925 6690/0409 521 234.

Applicants are reminded that the research may only commence upon receipt of formal written approval from the Animal Ethics Committee. Whilst all attempts are made to provide as timely a response as possible, please allow a minimum of 30 working days from the submission of your application to receipt of a first written response from the Animal Ethics Committee. Further information can be found on Animal Ethics website at: http://www.rmit.edu.au/staff/research/research-integrity-and-governance/animal-ethics, or contact the Ethics Officer at: animalethics@rmit.edu.au.
APPLICATION FORM

1. Project Details

1.1 Project Title

The title of the project should be concise and expressed in lay language. Avoid abbreviations and scientific jargon.

Use of genetically deficient and wild-type mice for the identification of novel therapeutics for Chronic Obstructive Pulmonary Disease

1.2 Principal Investigator

The Principal Investigator has ultimate legal responsibility for the welfare of all animals used

Title & Full Name: A/Prof Ross Vlahos
School/Institute: School of Health Sciences, Health Innovations Research Institute

1.3 Duration of Project Approval

Applicants may request approval for a project for up to three (3) years, which is subject to annual review. If the application is approved, you will be advised of the AEC-approved start and end dates on the formal written approval notice.

Proposed project commencement date: 30/11/2015
Proposed project conclusion date: 30/11/2018

1.4 Indicate the Scientific Procedures Premises/Fieldwork Licence (SPPL/SPFL)

- SPPL50 - RMIT Applied Sciences
- SPPL365 - RMIT Health Sciences
- SPPL302 - RDDT for RMIT Drug Discovery Technologies Pty Ltd
- SPFL40 - RMIT Life & Physical Sciences
- SPPL49 - RMIT School of Medical Sciences

1.5 Is this project a continuation of a previously approved project?

- No
- Yes

1.6 Project Type

- Diagnosis
- Product Testing
- Production of Biological Products
- Research
- Teaching - Undergraduate/Postgraduate
- Training in Procedural Techniques
- Other

1.7 Use of Genetically Modified Organisms (GMO)

Does this project require approval from RMIT's Institutional Biosafety Committee (IBC)?

- No
- Yes

What is the background strain: C57BL/6
Have you provided the relevant Animal Facility Manager the relevant health profile of the source colony?

[X] No   [ ] Yes

Briefly describe the function(s) of the gene(s) that have been or will be modified?

- The NADPH oxidase (NOX)-1 gene encodes for a protein that is highly expressed in epithelial cells in the lung and other tissues such as the colon and vasculature. NOX-1 is the major component of an enzyme complex that generates toxic Reactive Oxygen Species (such as superoxide) in response to invading pathogens and cigarette smoke. We believe that NOX-1 is up-regulated in COPD and other lung inflammatory conditions and is responsible for generating Reactive Oxygen Species that damage the lung.

- The NOX-2 gene encodes for a protein that is highly expressed in professional phagocytes such as neutrophils and macrophages that generate large amounts of superoxide as part of their armoury of bactericidal mechanisms. Although NOX-2 containing oxidases intentionally generate Reactive Oxygen Species for functions related to innate immunity and signal transduction, we believe that NOX-2 is up-regulated in COPD and other lung inflammatory conditions, which is responsible for generating unusually high levels of Reactive Oxygen Species that ultimately damage the lung.

- The NOX-4 gene encodes for a protein that is highly expressed in epithelial and endothelial cells in the lung. NOX-4 is homologous to NOX-1 and NOX-2 and is an important component of an enzyme complex that generates toxic Reactive Oxygen Species such as hydrogen peroxide in those cells. Whilst NOX-4 expression is generally low, we believe it is up-regulated in COPD and other lung inflammatory conditions and responsible for generating reactive oxygen species that damage the lung.

- Glutathione peroxidase-1 (Gpx-1) is an endogenous anti-oxidant enzyme that catalyses the potentially toxic hydrogen peroxide to water and oxygen. The mice that lack Gpx-1 have higher levels of oxidative stress owing to a reduced capacity to clear hydrogen peroxide.

- Interleukin-17 (IL-17) is a cytokine produced at local sites (in particular immune cells called T cells) within the body.

- NOX-1: Our microarray and proteomic studies have shown that a number of oxidative stress molecules are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease. We believe that NOX, the enzyme responsible for generation of lung destroying Reactive Oxygen Species (ROS), may be a novel therapeutic target for COPD and Acute Exacerbations of COPD, as it is upstream of other anti-oxidants.
Explain the relevance of this genetic alteration to the project?

(eg superoxide dismutase). The NOX-1 gene encodes for a protein that is highly expressed in epithelial cells in the lung. We propose that mice deficient in NOX-1 may be protected from cigarette smoke-induced lung injury because these mice are incapable of producing lung destroying Reactive Oxygen Species in response to LPS and cigarette smoke. Thus, we now wish to explore whether animals deficient in NOX-1 are protected from cigarette smoke-induced lung inflammation. In all of our proposed experiments we will use mice at 7 weeks of age, an age where baseline phenotypes would unlikely severely impact on the well-being of the animals.

-NOX-2: Our microarray and proteomic studies have shown that a number of oxidative stress molecules are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease. We believe that NADPH oxidase (NOX), the enzyme responsible for generation of lung destroying Reactive Oxygen Species (ROS), may be a novel therapeutic target for COPD and Acute Exacerbations of COPD, as it is upstream of other anti-oxidants (eg superoxide dismutase). The NOX-2 gene gene encodes for a protein that is highly expressed in cells of the lung and blood (neutrophils and macrophages). We propose that mice deficient in NOX-2 may be protected from cigarette smoke-induced lung injury because these mice are incapable of producing lung destroying Reactive Oxygen Species in response to cigarette smoke. Thus, we now wish to explore whether animals deficient in NOX-2 are protected from cigarette smoke-induced lung inflammation.

-NOX-4: Our microarray and proteomic studies have shown that a number of oxidative stress molecules are involved in the pathogenesis of COPD. We now believe that NADPH oxidase (NOX), the enzyme responsible for generation of lung destroying Reactive Oxygen Species (ROS), may be a novel therapeutic target for COPD, as it is upstream of other anti-oxidants (eg superoxide dismutase). The NOX-4 gene encodes for a protein that is highly expressed in epithelial and endothelial cells in the lung. We propose that mice deficient in NOX-4 may be protected from cigarette smoke-induced lung injury because these mice are incapable of producing lung destroying Reactive Oxygen Species in response to cigarette smoke. Thus, we now wish to explore whether animals deficient in NOX-4 are protected from cigarette smoke-induced lung inflammation.

-Gpx-1: The ability to alter the genome of the mouse gives the researchers an insight into the importance of the gene, in this case, the role of Gpx-1 in the effect of COPD.
How much is known about the biological characteristics/phenotype of this strain? (Select an option)

- Well characterised [X]
- Partially characterised/some information available
- Unknown

Describe how the animal health, welfare, breeding or lifespan will be affected (or the potential effects) by this genetic modification?

- NOX-1: The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.
- NOX-2: The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.
- NOX-4: The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan.
- Gpx-1: The mice that lack Gpx-1 are under oxidative stress owing to a reduced capacity to clear hydrogen peroxide. It has been reported Gpx-1 deficient mice demonstrated increased inflammation and cell death following a cold-induced brain injury or cerebral ischemia. At 8 months of age Gpx-1 deficient mice weigh 20% less than wild-type controls but no differences were detected in liver, kidney or heart tissue weights compared to wild-type controls. Gpx-1 deficient mice have no known histopathological or developmental abnormality - they are healthy and fertile (longest reported study up to 15 months of age) consistent with a limited role for gpx-1 during normal development and under physiological conditions (Ho et al, 1997; Cheng et al 1997; de Haan et al 1998).
- IL-17: The animal has no behavioural, physiological, reproductive and developmental features that affect its health, welfare, breeding or lifespan. IL-17 deficient mice have a normal physical appearance and breed normally. These mice only display impaired immune responses if subjected to certain experimental challenges (e.g. lung asthma models) (Nakae et al. 2002. Antigen-Specific T Cell Sensitization Is Impaired in IL-17-Deficient Mice, Causing Suppression of Allergic Cellular and Humoral Responses Immunity 17: 375-387).

Describe the breeding program that will be used to produce the genetically modified animals.

Does the strain require any special husbandry?

- No [X]
- Yes
1.8 Permits/Licences/Approvals

Are Permits Or Licences Other Than The Scientific Procedures Premises Licence (SPPL) Required (Eg. DSE Wildlife Research Permits, Interstate Licences)?

[ ] No  [ ] Yes

2. Aims and Significance of Project (Code of Practice 2.216 [vi & xi])

2.1 General Background Details

Please provide a BRIEF discussion of the background of the project (e.g. is this project part of a larger research programme?) Must be in PLAIN ENGLISH. Avoid or DEFINE scientific terms. This section should provide AEC members, particularly external lay or welfare members who have no scientific background with a clear idea of the potential benefits and significance of the project.

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease that affects millions of people worldwide with an annual cost of over $800 million in Australia alone. Currently there is no effective therapy to treat people with this debilitating disease, which is predicted to become the 3rd leading cause of death worldwide by 2020.

COPD is characterised by increased lung inflammation, leading to an irreversible air flow limitation that causes coughing and shortness of breath. The inflammation in the lungs is caused by inhalation of irritants that damage the lungs; the primary cause is inhalation of cigarette smoke, either directly through smoking, or via inhalation of secondhand smoke from the air in an environment where other people are smoking (i.e. passive smoking). Other irritants that can contribute to COPD are air pollution, chemical fumes and dust particles from the environment or workplace.

COPD is a progressive disease, worsening over time and causing damage to the lung structure and enlargement of the airspaces (emphysema). In addition to elevated inflammation and immune cells within the lungs, COPD is also associated with a high oxidant burden both from the oxidants contained within cigarette smoke and also from oxidative chemicals released from activated immune cells.

As the disease progresses, patients with COPD experience a range of debilitating systemic effects including skeletal muscle wasting and cardiovascular dysfunction. Skeletal muscle loss (muscle wasting) is experienced by up to 40% of COPD patients and is a strong independent risk factor for death, severely reduces the patient’s quality of life and increases disease burden and the incidence of hospitalisation. Increased lung and systemic inflammation and oxidative stress are thought to be contributing factors underlying the skeletal muscle wasting and cardiovascular pathologies seen in COPD patients. Our research aims to understand the mechanisms by which inflammation and oxidative stress lead to COPD, muscle wasting and other associated pathologies, with the goal of identifying key molecules involved in disease progression that could be targeted through novel therapies to help treat COPD patients.

Over a period of 15 years we have developed and established mouse models of COPD that involve exposing mice to low concentrations of cigarette smoke (University of Melbourne AEEC #s 02084, 02085, 05097, 05107, 06199, 0810912, 1011596, 1112185, 1312807, 1312736). We have shown that these models replicate many of the features of human disease including lung inflammation, emphysema (air space enlargement) and skeletal muscle wasting. Our previous work has added to the body of knowledge about the factors involved in COPD development and progression, and we have identified a number of genes and proteins that are likely involved in mediating the effects of COPD and could be potential therapeutic targets for treating this debilitating disease. Our current research aims to further investigate the role of oxidative stress and inflammation in COPD and its associated comorbidities, and to expand on our recent findings to further develop novel therapies for COPD.

2.2 Aims

Clearly and concisely describe the aim(s) of the project (preferably in bullet points)

The aims of this project are to:
- Further understand the role of oxidative stress and chronic inflammation in the development and pathology of COPD both

Page 6 of 35
within the lung environment and systemically.
- Identify genes and proteins that could be targeted therapeutically to prevent, delay or reverse the effects of COPD or its comorbidities (muscle wasting and cardiovascular changes)
- Evaluate the role of genes identified in our previous studies in the pathology of COPD by assessing the effects of cigarette smoke exposure in mice genetically deficient in these genes.
- Evaluate the effectiveness of existing and novel therapeutic agents (e.g., small molecules and neutralising antibodies) in reducing oxidative burden and disease severity using our mouse models of COPD.

3. Justification for Animal Use & the three Rs (Code of Practice 2.2.16 [xi])

3.1 Animals to be Used

Please ensure you include ALL animals used, e.g., embryos, neonates, pregnant animals and any expected by-catch in trapping projects. An animal is any live, non-human vertebrate, that is, fish, amphibians, reptiles, birds, and mammals, encompassing domestic animals, purpose-bred animals, livestock, wildlife, and also cephalopods such as octopus and squid.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of Animals (total)</th>
<th>Source and Permit</th>
<th>Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse - BALB/c males</td>
<td>1848</td>
<td>Animal Resource Centre - Western Australia</td>
<td></td>
</tr>
<tr>
<td>Mouse (Genetically Modified) - Gpx-1 deficient and Wild-Type males</td>
<td>216</td>
<td>University of Melbourne</td>
<td></td>
</tr>
<tr>
<td>Mouse (Genetically Modified) - NOX-1 deficient and Wild-Type males</td>
<td>216</td>
<td>Monash University</td>
<td></td>
</tr>
<tr>
<td>Mouse (Genetically Modified) - NOX-2 deficient and Wild-Type males</td>
<td>216</td>
<td>Monash University</td>
<td></td>
</tr>
<tr>
<td>Mouse (Genetically Modified) - NOX-4 deficient and Wild-Type males</td>
<td>216</td>
<td>Monash University</td>
<td></td>
</tr>
<tr>
<td>Mouse (Genetically Modified) - IL-17 deficient and Wild-Type males</td>
<td>216</td>
<td>Hudson Institute of Medical Research (formerly Monash Institute of Medical Research)</td>
<td></td>
</tr>
</tbody>
</table>

Total number of animals to be used for this project: 2,928

3.2 Potential Benefit(s) of the Project (Code of Practice 2.2.16 [v])

What is the potential benefit to humans or animals as per the Code of Practice 1.1 which states that “Scientific and teaching activities using animals may be performed only when they are essential

- [x] to obtain and establish significant information relevant to the understanding of humans;
- [ ] to obtain and establish significant information relevant to the understanding of animals;
- [x] for maintenance and improvement of human health and welfare;
- [ ] for maintenance and improvement of animal health and welfare;
3.3 Referring to the Potential Benefits above, summarise the scientific/environmental and/or educational significance and potential benefit(s) of the project (including its relation to previous studies)

The studies outlined in this proposal will contribute significantly to our understanding of the mechanisms underlying COPD, with a particular focus on the roles of oxidative stress and chronic inflammation in the progression of both the lung pathology and systemic comorbidities (muscle wasting and cardiovascular pathology). This will allow us both to identify key molecules that could be targeted therapeutically, and to test the effectiveness of existing and novel pharmacological agents in treating the disease in our animal models.

3.4 Replacement (Code of Practice 2.2.16 [viii])

This section requires you to justify WHY you think you need to use animals in this project. Are there alternatives to using animals in this project? Replacement is defined as the substitution of insentient material for live and conscious higher-order animals.

The Code specifies that techniques that totally or partially replace the use of animals for scientific purposes must be sought and used wherever possible. Suitable websites and databases that could be useful include:

http://altweb.jhsph.edu/resources
www.nal.usda.gov/awic/alternatives/alternat.htm

3.4.1 Provide details of the search conducted to find alternatives to the use of animals for this project. Include a list of the websites and databases visited (and the dates visited)


3.4.2 Can experiments be conducted on isolated cells, tissues and organs or non-recovery-anaesthetised animals?

X No ☐ Yes

Explain why not

The complex inflammation that triggers COPD reactions cannot be modeled using computers, nor can cell culture techniques address questions involving integrated pathophysiology. The experiments proposed in this application cannot be ethically performed in humans. The genes and proteins that regulate inflammation in the mouse are highly conserved in humans making the findings we obtain directly relevant to understanding human disease. Other members of the laboratory (A/Prof Steven Bozinovski) have cell culture systems where certain cells known to be involved in COPD (e.g. epithelial and macrophages) are exposed to cigarette smoke extract. These studies have provided us with valuable information but are limited as they cannot replicate in vivo biology and only deals with the water-soluble components of cigarette smoke. We have designed our experiments so as to limit the number of animals used whilst obtaining maximum information.

3.5 Reduction (Code of Practice 2.2.16 [vii])
Reduction refers to using the minimum number of animals and the most appropriate species/strain/age/sex, whilst obtaining data of sufficient volume, precision or statistical significance. Justification must be given for the number of animals used, ensuring that research/teaching activities are not repeated unnecessarily, whilst adequate numbers of animals are used to ensure scientifically sound and useful data is obtained.

3.5.1 What is the basis for selection of the species/strain/sex and age requested?

The genes and proteins that regulate inflammation in the mouse are highly conserved in humans making the findings we obtain directly relevant to understanding human disease. In addition, gene modification technology in the mouse will be used in this application to extend the information gained from the microarray studies in the same species. Moreover, mice are commonly used for these types of experiments due to the accessibility of the mouse genome, ready availability of commercial antibodies and other immunological reagents and the investigators have extensive experience in this species. Balb/c mice (7 weeks of age) were chosen for this series of experiments as the models being utilized in this study were developed in these mice. We have chosen to use male mice for this study due to the hormonal effects causing differences in the regulation of bodyweight and muscle mass between males and females. We also would like to conduct experiments on the various knockout mice as we have learned that removing the genes listed in the application will potentially prevent inflammation and COPD compared to the WT counterparts.

3.5.2 Justify the number of animals requested, in terms of the statistical and/or non-statistical considerations used in the study design

The group size of 12 per group (8 [biochemistry] + 4 [histology]) has been based on power calculations to ensure a maximum likelihood of detecting real differences in parameters between control and treated mice based on historical variances in our laboratory for similar protocols (University of Melbourne AEEC # 02084, 02086, 05097, 05107, 06199, 0810912, 1112185, approx. 15 years). The experiments have been designed to extract the maximum information from as few mice as possible. While the animal numbers listed in the attached document seem large, each experiment has been designed to produce definitive outcomes preventing possible repeats of experiments.

3.5.3 Has a Statistician/Biometrician been consulted about the design of this project?

☐ No ☑ Yes

Please explain why this was not considered necessary

Our laboratory has over 15 years experience in performing animal experimentation in accordance to the requirements of the animal ethics committee to ensure that the minimal number of mice are used without compromising the experimental outcomes. Also see response 3.5.2 above.

3.5.4 Repeated Studies

Does this project duplicate work that has been conducted previously?

☐ No ☑ Yes

3.5.5 Can tissues be shared with/acquired from another project or researcher?

☐ No ☑ Yes
Please provide details

Most of the tissues in the mice will be harvested during this project due to the long-term nature of the protocol. The tissues will be from mice that have been treated with cigarette smoke and pharmacological agents and so may not be suitable for the research purposes of other researchers. However, tissues not required for our experiments will be available any students, or researchers who might have need for them, provided they are suitable for those needs.

3.6 Refinement (Code of Practice 2.2.16 [vii])

Refinement refers to any techniques/modification/design specifications instituted to decrease, minimise and where possible, avoid any negative welfare impacts of procedures conducted on animals.

3.6.1 How has this project been designed to refine animal welfare?
Examples include provision of analgesia and project design to minimise number procedures conducted on individual animals.

We have chosen the lowest doses of pharmacological agents and minimised the number of doses given to each animal (depending on the agent) in order to achieve statistically significant effects.

3.6.2 If this is a disease/injury model, include a description of how the model works, effects of the progression of the disease process and identify humane end-points and previous experience with the model

Our cigarette smoke exposure protocols model various aspects of COPD in a mouse model. The model works in the same way as cigarette smoke exposure causes COPD in humans, through inhalation of cigarette smoke into the lungs. The model progresses from a mild lung inflammatory response (from acute exposures to smoke) and progresses to a more chronic inflammatory state with damage to the lung structure (emphysema) and associated pathologies including muscle wasting arising from long term smoke exposure. We have developed and used this model for the last 15 years and have not experienced an adverse side effects or events from the exposure of mice to cigarette smoke for periods of up to 6 months.

On arrival into the RAF from the relevant source (eg ARC), mice will be ear-tagged by RAF staff for identification purposes. We will ensure that health reports will be provided to the RMIT Animal Facility prior to animals entering the facility. Mice will then be organised into weight matched groups and acclimatised to their new location for 1 week, after which the experimental period will begin.

Treatment with Pharmacological Agents:

This project focuses on reducing inflammation and oxidative stress as a potential therapeutic approach for treating COPD. To fulfill this aim, mice will be treated with agents that have antioxidant functions, block the actions of mediators that cause inflammation or regulate oxidative stress and reactive oxygen species production/metabolism. The agents to be used will depend on the mediator we wish to block and its reported mode of action. Our previous studies (University of Melbourne AEEC# 00176, 02085, 05097, 05107, 06199, 0810912, 1011596, 1112185) have shown that the mediators found in increased amounts in smoke-exposed lung compared to control (sham) can be blocked by using (i) small molecular weight compounds or (ii) neutralising antibodies.

The dose of agent to be used will be obtained from the literature or provided by pharmaceutical companies and will be used in the range known to be safe and well tolerated by mice. In designing small molecular weight compounds, each company has performed detailed pharmacokinetic analyses which will allow us to use effective doses at blood and target tissue. Since they are developmental compounds they have undergone rigorous acute (single rising dose) and 1 week protocols (sub-chronic dosing).
In addition we know from previous studies that the drugs are very well tolerated. Some of the companies have also developed a range of monoclonal neutralising antibodies that are safe over wide dose ranges. Monoclonal antibodies are used for larger molecular weight mediators where small molecular weight compounds do not work or have not been identified.

The class and mode of action of the types of drugs to be used include:

i) Small molecular weight compounds: Apocynin (5mg/kg intraperitoneal injection), Ebselen (10mg/kg oral gavage), Nitrooxide (30mg/kg IP injection), Milfasartan (30mg/kg IP injection), Nitrosartan (30mg/kg IP injection)

ii) Neutralising antibodies (eg against GM-CSF, IL-17A): 0.1-5mg/kg, via Intranasal, Intraperitoneal injection, Subcutaneous injection or intravenous injection routes.

Depending on the property of the mediator in question the agents will be administered in a variety of ways including oral gavage, intraperitoneal injection, subcutaneous injection, intravenous injection or intranasal administration. Each individual animal will receive one form of test agent by one route of administration only. Depending on the route of administration each animal will not receive more than 1 oral gavage, 2 intraperitoneal injections, 1 intranasal administration, 1 intravenous administration or 1 subcutaneous injection in a 24 hour period. Due to the nature of the agents we cannot state the exact time intervals between each procedure since many of the test agents have different pharmacokinetic profiles (the time period that the drug works). The administration methods to be used are as follows:

**Oral gavage:** Compounds will be administered once daily by oral gavage in a dose range between 0.2-200 mg/kg. Animals will be restrained and a gavage tube will be used to push the head slightly upward and back to straighten the oesophagus. The tube will be positioned to the right or left of the mouth and inserted slowly while watching for the swallowing reflex. The tube will pass freely into the oesophagus, without being forced. When the desired length of insertion is achieved, the compound solution will be dispensed (no needle is required, volume used will be 100-200 μl per treatment). Mice will then be observed after the procedure for any signs of distress. While some experiments will use only an acute smoke exposure protocol (4 days), to observe the full COPD profile including air space enlargement (emphysema) and muscle wasting we will use longer term models (6 months, 5 days per week). Therefore, in these instances it is necessary to treat mice by oral gavage daily for the duration of the 6 month protocol to determine whether cigarette smoke-induced lung damage (i.e. emphysema) and muscle wasting is prevented or reduced.

**Intraperitoneal (IP) injections:** Injections will be administered to conscious mice using a 27 gauge needle on a 1 ml syringe. The volume of compound will be no more than 300 μl. IP injections will be administered using a small volume (maximum 200μl) of drug using a 27 gauge needle will be injected just under the pinched skin.

**Subcutaneous injection:** Mice will be flattened slightly against a smooth stable surface and the skin around the shoulders will be lightly pinched. A small volume (maximum 200μl) of drug using a 27 gauge needle will be injected just under the pinched skin.

**Intranasal administration:** This method ensures a concentrated dose of agent directly into the lung. Mice will be lightly anaesthetized with the inhalation anaesthetic methoxyflurane as described below and a small amount (50 μl) of solution containing the pharmacological agent will be applied to the nostrils once per day of treatment (up to 14 days). Anaesthetizing the animals allows the experimenter to treat the animal quickly and effectively as they are immobilised and as mice are obligate nose breathers the treatment agent will be distributed uniformly throughout the lung. We have routinely used methoxyflurane for the last 15 years for this reason, and in our experience this procedure is well tolerated by the mice without observed adverse effects. In addition, there are studies showing that inhaled isoflurane inhibits LPS-induced lung inflammation in mice (Marcias et al 2010 Inhaled Isoflurane In Mice Ameliorates LPS- and Ventilator-Induced Lung Injury; Reutershan et al. 2006 Protective Effects of Isoflurane Pretreatment in Endotoxin-induced Lung Injury) and we have recently found that inhaled isoflurane inhibits cigarette smoke-induced lung inflammation. For these reasons, inhaled isofoxane cannot be used in our experiments.

**Intravenous administration (via tail vein):** Mice will be warmed by placing them in a cage under a custom-built heat lamp apparatus (owned by the BRF) for a few minutes. Mice will then be removed from the cage and then restrained (without sedation), using a purpose-built mouse restrainer and a small volume (50 μl) of compound will be injected, using a 30 gauge needle, into the mouse tail vein, once per day of treatment (up to 14 days). The injection site will be rotated so that the top tail vein is used one day and the bottom tail vein is used the next day.

Please note that all of these procedures will only be performed by experienced investigators.

Acute Cigarette smoke exposure (4 days):
4. Animal Experience and Impact on Each Individual Animal

4.1 Provide a brief overview of the experimental design and procedures to be performed on animals

Mice are put into an 18 litre plastic container connected to a 3-way tap where cigarette smoke will be pumped. A cigarette is connected to the tap (using a pipette tip) along with a 60 ml syringe, which is then used to draw smoke from the lit cigarette and push it into the chamber. It takes ~3 min to smoke 1 cigarette using a constant rate of 10 sec to draw up the syringe barrel. The box lid is left on for another 12 min and then removed for 5 min of fresh air. This is done 3 times in succession 3 times per day, ie the maximum smoke exposure is 9 cigarettes per day over at least an 8 hour period. Assay for carboxyhaemoglobin in blood has shown that smoke inhalation in mice is similar to mild human smoking, and histology has shown that we are not causing acute lung injury. Over a 14 day smoke protocol, mice lose around 7% of their body weight but there is no reduction in activity and weight is rapidly regained once smoking stops. 4 days of smoke produces many features of COPD, including an increase in numbers of key types of white blood cells in the lung and increased lung protease activity. Each experiment will have a matched (exposure period) sham smoked group of animals that will be placed in the perspex chamber but not exposed to cigarette smoke. Similar experiments will also be performed in genetically modified mice to determine the importance of defects of certain genes in the development of COPD.

In previous studies mice from different experimental groups (drug treated vs untreated) have been exposed to smoke simultaneously within the same 18L plastic container, and we have not experienced problems with mice fighting or becoming distressed during this period. However, in the event that fighting or other problems do occur during the smoking period, a divider will be used to split the smoke box and separate the groups, allowing them to be exposed to the same smoke but preventing them from contacting other groups.

Previous experiments (University of Melbourne AEEC#s 02084, 02085, 05097, 05107, 06199, 0810912, 1112185) have shown this to be observably painless and causing minimal observable discomfort to the animal. However, in the event the animals show signs of distress (animals will be monitored daily using attached monitoring and intervention checklists) such as loss of body weight (more than 10% over 24 hours period), changes in respiration, lethargy, piloerection, hunched appearance, loss of appetite and loss of grooming, they will be immediately killed by an overdose (intraperitoneal injection) of anesthetic (sodium pentobarbitol - 240mg/kg). A 1 ml syringe with a 27 gauge needle will be used for this injection. Death will be ensured by opening of the chest cavity.

Chronic Cigarette smoke exposure (6 month protocol):

Whilst the acute 4 day cigarette smoke exposure produces many of the features of COPD, we have shown that longer term models (up to 6 months) are required to observe the full COPD symptom profile including air space enlargement (emphysema) and skeletal muscle wasting. For this model we propose to expose mice to cigarette smoke in the same fashion as the 4 day smoke protocol, however rather than exposing mice to 9 cigarettes per day we propose 6 cigarettes per day (2 in the morning, 2 at noon, and 2 in the afternoon) – the maximum length of each exposure is 40 minutes. Data from previous long-term smoking protocols (6 cigarettes/day, 5d/week for 4 weeks or 6 months) show that mice do not lose weight but gain less weight than no-smoke control mice. Each experiment will have a matched (exposure period) sham smoked group of animals that will be placed in the Perspex chamber but not exposed to cigarette smoke. Similar experiments will also be performed in genetically modified mice to determine the importance of defects of certain genes in the development of COPD.

Previous experiments (University of Melbourne AEEC #02085, 0810912, 1112185) have shown that there is minimal discomfort to the animals with long term exposures to cigarette smoke. Animals will be monitored daily using the attached monitoring sheet and intervention checklists) and in the event the animals show signs of distress such as loss of body weight (more than 10% over a 24 hour period), changes in respiration, lethargy, piloerection, hunched appearance, loss of appetite and loss of grooming, they will be immediately killed by an overdose (intraperitoneal injection) of anaesthetic (sodium pentobarbitol - 240mg/kg). A 1 ml syringe with a 27 gauge needle will be used for this injection. Death will be ensured by opening of the chest cavity.

Pair Feeding:

For the Chronic cigarette smoke exposure protocols (1 and 6 months) it will be important to include a pair-fed control group, which will receive the exact amount of food eaten by the relevant experimental group (e.g. smoke-exposed mice) on the previous day. This is important as nicotine (contained in cigarette smoke) has been shown to cause a small reduction in food
intake, which may impact on the regulation of muscle mass. Exposure to smoke causes a small reduction in food intake which we have shown previously (AEC # 02084, 06237) to reduce body weight over the course of 4 month experiment by approximately 10%. This small restriction of food does not appear to distress the pair-fed animals. By including pair-fed control groups it will be possible to determine the relative contribution of reduced food intake to the observed changes and therefore the effect of cigarette smoke exposure itself on changes in muscle mass and pathology. Animals will be monitored daily and in the event an animal shows signs of distress they will be immediately killed by an intraperitoneal anaesthetic overdose administered via a 27 gauge needle (see below and attached monitoring sheet).

Animal monitoring:

Mice will be monitored daily for signs of illness such as loss of body weight, lethargy, ruffled fur, hunched appearance, panting, loss of appetite (as seen by monitoring food intake), isolation from the group and loss of grooming (ie criteria set out in attached monitoring and intervention checklists). Weights will be recorded daily in all experiments except for the 6 month smoke studies in which weights will be recorded on Monday, Wednesday, Friday. Food intake will also be measured by weighing the food three time a week (Monday, Wednesday, Friday). Mice will be observed daily during the experimental protocol to ensure that no overt lung responses, respiratory distress or discomfort are elicited by cigarette smoke exposure. If an adversely severe reaction occurred the mice would show reduced mobility, panting and piloerection - in such circumstances they would be immediately killed by anaesthetic overdose (sodium pentobarbitol - 240mg/kg). Death will be ensured by opening of the chest cavity.

At the conclusion of the experiment:

At the end of the experimental protocol, mice will be killed by an intraperitoneal anaesthetic overdose (sodium pentobarbitol - 240mg/kg) administered via a 27 gauge needle and subjected to the procedures described below. Death will be ensured by opening of the chest cavity.

Procedures after the animals have been killed:

1. Blood samples will be taken from the inferior vena cava of each animal and analysed to determine signs of systemic inflammation.

2. The diaphragm is opened, lungs lavaged (method to obtain fluid from the lungs to determine level of inflammation) with PBS (400 μl, followed by 3 times 300 μl aliquots) via the trachea. A separate group of animals (n=4 per experimental group) will have their lungs inflated with 10% neutral buffered formalin at a pressure of 20cm of mercury for 10 minutes, to fix the lung tissue for later histological examination. These two procedures cannot be performed on concurrently on the same animals.

3. The chest cavity is opened and lungs removed and examined for inflammatory changes using (i) Cellular inflammation (principally lavage); (ii) RNA extraction for gene profiling using gene microarrays/QPCR and (iii) histology and immunohistochemistry. These procedures cannot be concurrently performed in the same animal and the experiments have been designed to extract the maximum information from as few mice as possible.

4. Organs such as the liver, kidney, fat pads, skeletal muscles from the hind limb (gastrocnemius, soleus, tibialis anterior, plantaris), heart, spleen, brain and ilium will be dissected, weighed and snap frozen for subsequent analysis. This will include (i) RNA extraction for gene profiling, (ii) determination of protein levels and (iii) histology and immunohistochemistry.
## 4.2 Impact on the animals

Do experiments involve (please tick) *ANIMAL USE CATEGORIES*

<table>
<thead>
<tr>
<th>ANIMAL USE CATEGORIES</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate euthanasia of animals to obtain tissue for biochemical analysis, or in vitro, cell, tissue or organ studies.</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Experiments under anaesthesia, without recovery (i.e. animals are fully anaesthetised for the duration of the experiment, and are killed at its conclusion without recovery from anaesthesia).</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>No anaesthesia, minor procedures used (e.g. injections, blood sampling, antibody raising, minor dietary manipulations.)</td>
<td>☐</td>
<td>☒</td>
</tr>
<tr>
<td>Survival after an intervention which causes minor stress of short duration (eg. following biopsies or cannulations).</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Survival after an intervention which causes major or prolonged stress (eg. major surgery, exposure to heat, cold, ionising radiation; administration of toxic drugs; genetic manipulation, induction of neoplasia or foetal intervention.)</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Experiments under anaesthesia, with recovery.</td>
<td>☐</td>
<td>☒</td>
</tr>
<tr>
<td>Observation of free roaming animals only</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Anaesthesia in non-experimental procedures/off protocol procedures (e.g. microchipping for identification)</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Other- please describe:</td>
<td>☒</td>
<td>☐</td>
</tr>
</tbody>
</table>

## 4.3 Please indicate if the project involves any of the following?

<table>
<thead>
<tr>
<th>Activity</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death as an end point (as defined in the Code)</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Production of monoclonal antibodies by ascites method</td>
<td>☒</td>
<td>☐</td>
</tr>
<tr>
<td>Prolonged restraint or confinement (e.g. CLAMS)</td>
<td>☒</td>
<td>☐</td>
</tr>
</tbody>
</table>

## 4.4 What is the maximum period of time that an individual animal or group of animals will be used in this project?

6 months
4.5 Complete the following summary table for ALL procedures to be performed on animals, including killing, both from protocol in emergency circumstances

Using the following table, separately identify and specify EACH procedure used in this proposal that may impact an animal’s well-being. This assessment is a step-by-step description of what will happen each animal. State how adverse effects will be minimised. List these procedures chronologically:

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Name of procedure</th>
<th>SOP Reference (if applicable)</th>
<th>Number of Animals</th>
<th>Timing and Duration of procedure</th>
<th>Location where this procedure will be conducted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expose mice to cigarette smoke</td>
<td>SOP-smoke exposure</td>
<td>refer to attached sheet</td>
<td>up to 60 mins, 3 times daily for 4 days to 6 months.</td>
<td>Level 11 PC2 facility</td>
</tr>
</tbody>
</table>

**SOP-otherwise exposure**

**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**

Mice are placed in an 18-liter Perspex chamber in a standard fume hood and exposed to cigarette smoke. In cigarette smoke exposure experiments, mice are exposed to cigarette smoke generated using 3, 6 or 9 cigarettes/day for 4 days up to 6 months. Cigarette smoke is delivered three times per day at 8 AM, 12 noon, and 4 PM with 1-3 cigarettes spaced over 1 h, respectively. Sham-exposed mice were placed in an 18-liter Perspex chamber but did not receive cigarette smoke.

**What adverse welfare impacts are possible?**

Weight loss, respiratory changes.

**How will adverse impacts be addressed and minimised?**

Mice will be monitored during and after each smoke session (3 times per day) for signs of distress according to the attached intervention criteria sheet. Body weights are monitored throughout the study.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Name of procedure</th>
<th>SOP Reference (if applicable)</th>
<th>Number of Animals</th>
<th>Timing and Duration of procedure</th>
<th>Location where this procedure will be conducted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>SOP-intranasal treatment via Methoxyflurane anaesthesia</td>
<td>refer to attached sheet</td>
<td>5 min</td>
<td>Level 11 PC2 facility</td>
</tr>
</tbody>
</table>

**SOP-otherwise intranasal treatment**

**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**

Intranasal administration ensures a concentrated dose of agent directly into the lung. Animals will be lightly anaesthetised with the inhalation anaesthetic methoxyflurane to induce anaesthesia. A mouse will be placed in a 1L air-tight container padded with cotton wool containing 2ml methoxyflurane, covered with gauze to prevent the mice coming into direct contact with the anaesthetic agent. The mouse is observed until a relaxed breathing is observed and there is a loss of the righting reflex when the container is gently tilted. The mouse is then removed from the box for intranasal administration and allowed to recover. This procedure is carried out in a fume hood to avoid release of anaesthetic vapours into the room.

**What adverse welfare impacts are possible?**

This is a routine method well tolerated by the mice - the anaesthesia is light and the mice recover within a minute following removal from the anaesthetic chamber.

**How will adverse impacts be addressed and minimised?**

Mice will be maintained under anaesthesia for the minimum duration and allowed to recover in a well ventilated area. Mice will be observed and monitored during their recovery and not returned to their home cages until fully recovered.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Name of procedure</th>
<th>SOP Reference (if applicable)</th>
<th>Number of Animals</th>
<th>Timing and Duration of procedure</th>
<th>Location where this procedure will be conducted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intranasal administration of agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Page 15 of 35
### SOP Reference (if applicable) | Number of Animals | Timing and Duration of procedure | Location where this procedure will be conducted
--- | --- | --- | ---
SOP-intranasal treatment via Methoxyflurane anaesthesia | refer to attached sheet | 5 min | Level 11 PC2 facility

**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**

Intranasal administration ensures a concentrated dose of agent directly into the lung. Animals will be lightly anaesthetised with the inhalation anaesthetic methoxyflurane (see above), in a fume hood. Anaesthesia will be confirmed through loss of the “righting reflex” - tested by rocking or tipping the contained gently. Once animals are appropriately anaesthetised a 35μl solution containing the agent will be applied to the nostrils once per day. Mice will then be allowed to recover until they are active before being returned to their home cages.

**What adverse welfare impacts are possible?**

This is a routine procedure well tolerated by the mice.

**How will adverse impacts be addressed and minimised?**

Mice will be monitored following the procedure to ensure they recover quickly and do not show any signs of distress. They will not be returned to their home cages until they are fully recovered and moving around normally.

### Procedure

**Name of procedure** Oral gavage

**SOP Reference (if applicable) | Number of Animals | Timing and Duration of procedure | Location where this procedure will be conducted**
--- | --- | --- | ---
SOP-Oral Gavage in Mice | refer to attached sheet | 2 min | Level 11 PC2 facility

**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**

Compounds will be administered once daily by oral gavage. Mice will be restrained in the hand and a round-tip gavage tube (purpose-made) will be used to push the head slightly upward and back to straighten the oesophagus. The tube will be positioned to the right or left of the mouth and inserted slowly while watching for the swallowing reflex. The tube will pass freely into the oesophagus, without being forced. When the desired length of insertion is achieved, the compound solution will be instilled (volume used will be no more than 10 mL/kg per dose). Mice will then be observed after the procedure for any signs of distress.

**What adverse welfare impacts are possible?**

This is a well tolerated and minor procedure

**How will adverse impacts be addressed and minimised?**

Following gavage, mice will be observed after the procedure for any signs of distress and ongoing daily monitoring will continue using the attached monitoring sheet.

### Procedure

**Name of procedure** Subcutaneous injection

**SOP Reference (if applicable) | Number of Animals | Timing and Duration of procedure | Location where this procedure will be conducted**
--- | --- | --- | ---
SOP-subcutaneous injection of mice | refer to attached sheet | 2 min | Level 11 PC2 facility

**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**

To ensure a slow-release pharmacokinetic profile some of the test compounds will be administered via a subcutaneous injection. Briefly mice will be flattened slightly against a smooth stable surface and the skin around the shoulders will be lightly scuffed. Test agent (volume no greater than 20ml/kg) will be injected using a 27 gauge needle just under the skin layer.

**What adverse welfare impacts are possible?**

Very minor to no distress, possible minor irritation that is not long lasting
### Procedure

**Name of procedure** | Intravenous administration of agents  
--- | ---  
**SOP Reference (if applicable)** | Number of Animals | Timing and Duration of procedure | Location where this procedure will be conducted  
--- | --- | --- | ---  
SOP-intravenous administration in mice | refer to attached sheet | 5 minutes | Level 11 PC2 facility  
**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**  
Mice will be warmed by placing them in a cage under a custom built lamp apparatus for a few minutes to cause vasodilation and allow for easier access to tail veins. Mice are then removed from the cage and then restrained (without sedation), using a purpose-built mouse restrainer and a small volume (50 microlitres) of compound will be injected using a 30 gauge needle, into the mouse tail vein. Mice will be treated up to once per day (up to 14 days).  
**What adverse welfare impacts are possible?**  
Irritation at the injection site. Stress from being restrained.  
**How will adverse impacts be addressed and minimised?**  
The injection site will be varied each day (using top tail vein one day, bottom tail vein the next day) to minimise irritation from injections. To minimise stress the procedure will only be undertaken by experienced, trained investigators and will be conducted as quickly as possible to reduce stress to the mice. Mice will be observed following the procedure for signs of distress.

---

### Procedure

**Name of procedure** | Anaesthesia overdose via intraperitoneal injection  
--- | ---  
**SOP Reference (if applicable)** | Number of Animals | Timing and Duration of procedure | Location where this procedure will be conducted  
--- | --- | --- | ---  
SOP-Anaesthesia overdose via intraperitoneal injection | refer to attached sheet | At end of experiment, duration 2 min | Vlahos/Bozinovski laboratory (PC2 room)  
**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**  
At the conclusion of the experimental protocol, mice will be killed by an intraperitoneal anaesthetic overdose of anaesthetic (sodium pentobarbitol 240mg/kg) using a 27 gauge needle buffered in PBS). Secondary confirmation of death will be confirmed by performing a thoracotomy.  
**What adverse welfare impacts are possible?**  
**How will adverse impacts be addressed and minimised?**

---

### Procedure

**Name of procedure** | Intraperitoneal injection of agents  
--- | ---  
**SOP Reference (if applicable)** | Number of Animals | Timing and Duration of procedure | Location where this procedure will be conducted  
--- | --- | --- | ---  
SOP-Intraperitoneal injection of agents in mice | refer to attached sheet | 2 min, no more than twice in a 24h period | Level 11 PC2 facility  
**Description of procedure (if SOP not available). If this is a modified SOP, please specify changes**  
These injections will be administered to conscious mice in the right lower quadrant of the abdomen using a 27 gauge needle on a 1 ml syringe. The volume of compound will be no more than 300 μL.
What adverse welfare impacts are possible?  
Very minor to no distress, possible minor abdominal irritation that is not long lasting.

How will adverse impacts be addressed and minimised?  
The mice will be closely monitored during the short recovery period for signs of distress and ongoing daily monitoring will continue using the attached monitoring sheet.

Procedure

Name of procedure: Pair feeding

SOP Reference (if applicable): refer to attached sheet

Number of Animals: duration of experiment, up to 6 months

Timing and Duration of procedure: Level 11 PC2 facility

Location where this procedure will be conducted: refer to attached sheet

Description of procedure (if SOP not available). If this is a modified SOP, please specify changes: To control for the effects of cigarette smoke and other agents on appetite and food intake, separate groups of mice will be pair-fed for the duration of the study, being fed the same amount of food as the smoke/treated group ate in the previous 24h. Food intake is monitored by weighing the food.

What adverse welfare impacts are possible?  
This procedure is well tolerated and generally involves only a minor restriction on food intake. Some weight loss occurs.

How will adverse impacts be addressed and minimised?  
The mice will be regularly monitored and their bodyweight and condition assessed according to the attached monitoring and intervention sheets. We have not experienced any adverse effects of pair feeding in previous studies.

Add procedure  Remove procedure

4.6 Fate of Animals

What is the fate of the animals in the project? (Select an option)

Humanely killed.  
[□] No  [X] Yes

Is there an opportunity to provide other investigators with cadavers or tissue?  
[□] No  [X] Yes

Please provide details: Most of the tissues are collected for analysis at the end of the experiment, however certain tissues may be available to other researchers if needed.

Returned to the wild  
[□] No  [X] Yes

Other  
[□] No  [X] Yes

4.7 For wildlife projects please include details of the number of study sites and full address of sites

4.8 Does the project involve the capture or trapping of wildlife?
5. Monitoring of Animals

Investigators are responsible for monitoring the welfare of their animals (Code of Practice 2.2.26). This responsibility begins when an animal is allocated to the approved project and ends with the specified fate of the animal at the completion of the project. Unexpected incidents that impact on the welfare of any individual animal/group of animals MUST be responded to immediately and reported to the AEC via an Adverse Incident Report [need link].

All personnel identified in this section must be familiar with the criteria for monitoring the welfare of the animals, and maintaining monitoring records. Welfare monitoring checklists must be kept together with animals, accessible to all nominated personnel, animal facility staff, and the AEC if requested.

5.1 For Monitoring Purposes, Is This Purely An Observational Study?

No ☒ Yes □

Describe how animals will be monitored day to day (routine monitoring) and by whom

Once the animals arrive from the source (eg ARC) the experimental procedures will begin after one week as animals require sufficient time to acclimatise to their new environment. During this acclimatisation period, animals are monitored twice by the investigators and daily by the Biological Research Facility staff. As animals have not undergone any experimental procedures at this time there should be no signs of distress; however we will monitor to determine if animals are fighting, and whether each animal looks to be in good condition.

During procedures, what specific signs will be monitored and how frequently?

During the procedures animals will be monitored daily for overt signs of distress including piloerection, decreased interaction with peers, loss of appetite (assessed by changes in body weight) using the attached monitoring checklist. In addition, body weight will be monitored every Monday, Wednesday and Friday in the 6 month studies.

After procedures, what specific signs will be monitored and how frequently?

After the procedures animals will be monitored daily for overt signs of distress including piloerection, decreased interaction with peers, loss of appetite (assessed by changes in body weight) using the attached monitoring checklist. In addition, body weight will be monitored every Monday, Wednesday and Friday in the 6 month studies.

5.2 Responsibility for Monitoring
WHO will be monitoring the animals under experimentation? The responsibility for monitoring animals under experimentation lies with the Principal Investigator.

A/Prof Ross Vlahos, A/Prof Steven Bozinovski, Dr Samantha Passey, Mr Huei Jiunn Seouw, Mr Ivan Bernardo

WHO will be responsible for emergencies and how will it be ensured that nominees can be contacted?

A/Prof Ross Vlahos

The nominee(s) can be contacted via mobile number or email.

The Animal Facility staff attend the Animal Facility daily. WHAT is the longest period the animals under experimentation will be left unchecked by the investigators (including weekends & Public Holidays)?

48h

Are Animal Facility staff to be asked to take any special responsibilities in addition to routine husbandry? If yes, staff must be named on the application in the Personal Declaration section.

No

What is the maximum time individual animals are to be held under experiment?

6 months

What is the maximum time individual animals are to be held in the animal facility?

7 months

5.3 Attach a copy of the monitoring checklist and record keeping form(s) you will use to record these observations, and label ‘Section 5.3’

5.4 Experimental Intervention/Endpoints/Euthanasia criteria

At what point would you intervene to kill or treat the animal to alleviate its pain or suffering? Specify in detail what clinical signs, e.g. change in behaviour, loss in body weight, tumour size, changes to normal feeding and drinking behaviour etc. you would use in making this decision.

Signs of distress are detailed in the attached intervention checklist and include including piloerection, decreased interaction with peers, loss of appetite (assessed by changes in body weight). Determination of whether an animal will be euthanized based on clinical and behavioral signs will be made by an experienced investigator and will depend on the severity of the symptom. Mice who display 1 or more of the “severe” signs on the attached Intervention Criteria Checklist or have any obvious signs of pain will be euthanized immediately with an overdose of anesthetic (sodium pentobarbitol - 240mg/kg) administered via a 27 gauge needle. Death will also be ensured by opening of the chest cavity.

Attach a copy of the clinical scoring sheet that will be used to determine intervention and label ‘Section 5.4’

6. Chemical & Biological Agents to Be Administered

6.1 Will chemical or biological agents be used/administered in the project? (including the vehicles in which active agents are to be delivered).

☐ No  ☑ Yes
<table>
<thead>
<tr>
<th>Agent Details</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name of agent</strong></td>
<td>Cigarette smoke</td>
</tr>
<tr>
<td><strong>Action</strong></td>
<td>Cigarette smoke triggers a mild inflammation in the lung and can lead to systemic inflammation</td>
</tr>
<tr>
<td><strong>Purpose of administration for this project (if different from action)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Dose rate (mg/kg)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>LD50 (if known)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Route of administration</strong></td>
<td>Indirect inhalation</td>
</tr>
<tr>
<td><strong>Duration of action</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Potential side effects of administration or withdrawal of the drug</strong></td>
<td>weight loss, appetite suppression</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agent Details</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name of agent</strong></td>
<td>Apocynin</td>
</tr>
<tr>
<td><strong>Action</strong></td>
<td>Inhibitor of the NADPH Oxidase (antioxidant effects)</td>
</tr>
<tr>
<td><strong>Purpose of administration for this project (if different from action)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Dose rate (mg/kg)</strong></td>
<td>5 mg/kg</td>
</tr>
<tr>
<td><strong>LD50 (if known)</strong></td>
<td>650 mg/kg (for mouse intraperitoneal injection)</td>
</tr>
<tr>
<td><strong>Route of administration</strong></td>
<td>Intraperitoneal injection once per day</td>
</tr>
<tr>
<td><strong>Duration of action</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Potential side effects of administration or withdrawal of the drug</strong></td>
<td>None reported</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agent Details</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name of agent</strong></td>
<td>Ebselen</td>
</tr>
<tr>
<td><strong>Action</strong></td>
<td>Glutathione peroxidase mimetic (antioxidant effects)</td>
</tr>
<tr>
<td><strong>Purpose of administration for this project (if different from action)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Dose rate (mg/kg)</strong></td>
<td>1-10 mg/kg</td>
</tr>
<tr>
<td><strong>LD50 (if known)</strong></td>
<td>6810mg/kg (mouse oral LD50)</td>
</tr>
</tbody>
</table>
### Agent Details

<table>
<thead>
<tr>
<th>Name of agent</th>
<th>Milfasartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Action</td>
<td>Angiotensin II receptor antagonist</td>
</tr>
<tr>
<td>Purpose of administration for this project (if different from action)</td>
<td></td>
</tr>
<tr>
<td>Dose rate (mg/kg)</td>
<td>1-30 mg/kg</td>
</tr>
<tr>
<td>LD50 (if known)</td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>Duration of action</td>
<td></td>
</tr>
<tr>
<td>Potential side effects of administration or withdrawal of the drug</td>
<td>None reported</td>
</tr>
</tbody>
</table>

### Agent Details

<table>
<thead>
<tr>
<th>Name of agent</th>
<th>Nitroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Action</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>Purpose of administration for this project (if different from action)</td>
<td></td>
</tr>
<tr>
<td>Dose rate (mg/kg)</td>
<td>1-30 mg/kg</td>
</tr>
<tr>
<td>LD50 (if known)</td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>Duration of action</td>
<td></td>
</tr>
<tr>
<td>Potential side effects of administration or withdrawal of the drug</td>
<td>None reported</td>
</tr>
</tbody>
</table>

### Agent Details

<table>
<thead>
<tr>
<th>Name of agent</th>
<th>Nitrosartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Action</td>
<td>Dual action pharmacophore - antioxidant and angiotensin II receptor antagonist</td>
</tr>
<tr>
<td>Purpose of administration for this project (if different from action)</td>
<td></td>
</tr>
<tr>
<td>Dose rate (mg/kg)</td>
<td></td>
</tr>
<tr>
<td>LD50 (if known)</td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td></td>
</tr>
<tr>
<td>Duration of action</td>
<td></td>
</tr>
<tr>
<td>Potential side effects of administration or withdrawal of the drug</td>
<td></td>
</tr>
<tr>
<td>Purpose of administration for this project (if different from action)</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Dose rate (mg/kg)</td>
<td>1-30 mg/kg</td>
</tr>
<tr>
<td>LD50 (if known)</td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>Duration of action</td>
<td></td>
</tr>
<tr>
<td>Potential side effects of administration or withdrawal of the drug</td>
<td>None reported</td>
</tr>
</tbody>
</table>

**Agent Details**

<table>
<thead>
<tr>
<th>Name of agent</th>
<th>Monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Action</td>
<td>Used to neutralise the actions of macrophage growth factors (e.g., GM-CSF) or mediators which sustain neutrophilic inflammation (IL-17).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purpose of administration for this project (if different from action)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose rate (mg/kg)</td>
<td>0.1 to 5mg/kg</td>
</tr>
<tr>
<td>LD50 (if known)</td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td>Intranasal, Intraperitoneal injection, Subcutaneous injection, intravenous injection</td>
</tr>
<tr>
<td>Duration of action</td>
<td></td>
</tr>
<tr>
<td>Potential side effects of administration or withdrawal of the drug</td>
<td>No adverse side effects observed based on historical experiences. Vehicle to be used will be sterile saline.</td>
</tr>
</tbody>
</table>

**Agent Details**

<table>
<thead>
<tr>
<th>Name of agent</th>
<th>Methoxyflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Action</td>
<td>Volatile inhalation anaesthetic agent</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purpose of administration for this project (if different from action)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose rate (mg/kg)</td>
<td>A mouse will be placed in a 1L air-tight container padded with cotton wool containing 2ml methoxyflurane, covered with gauze to prevent the mice coming into direct contact with the anaesthetic agent. The mouse is observed until a relaxed breathing is observed and there is a loss of the righting reflex when the container is gently tilted. The mouse is then removed from the box for intranasal administration and allowed to recover. This procedure is carried out in a fume hood to avoid release of anaesthetic vapours into the room.</td>
</tr>
<tr>
<td>LD50 (if known)</td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td>Inhalation</td>
</tr>
</tbody>
</table>
7. Source and Housing of Animals

7.1 Is this purely an OBSERVATIONAL study?

[X] No  [ ] Yes

Where will animals be sourced from? (Provide the name of the supplier)
Animal Resource Center, Perth; University of Melbourne; Monash University; Hudson Institute of Medical Research (formerly Monash Institute of Medical Research).

Where will the animals be housed?
RMIT Animal Facility

7.2 Have you consulted with the relevant Animal Facility Manager?

[ ] No  [X] Yes

7.3 Are the husbandry requirements for the animals described in an existing SOP?

[ ] No  [X] Yes

Please provide the housing SOP code:
RAF SOP1 Animal Users induction
RAF SOP40 Cage Stacking Policy

*Please discuss the housing requirements with the relevant Animal Facility Manager who will also provide the SOP code.

7.4 Describe how the species-specific behavioural needs of the experimental animals will be met in terms of the environmental enrichment that will be provided.

RAF SOP14 Environmental Enrichment

It is recommended that you consult with the relevant animal facility manager for assistance as SOPs for environmental enrichment may already have been developed for your species.

8. Personnel
8.1 List All Personnel Involved in Animal Use in the Project

**Personnel Details**

<table>
<thead>
<tr>
<th>Please specify role:</th>
<th>Principal Investigator</th>
<th>Co-Investigator</th>
<th>Other Staff (indicate):</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Title/Full Name</th>
<th>A/Prof Ross Vlahos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Email</td>
<td></td>
</tr>
<tr>
<td>Qualifications</td>
<td>PhD, University of Melbourne; Bachelor of Science (Honours), University of Melbourne</td>
</tr>
<tr>
<td>Department/Institution</td>
<td>School of Health Sciences</td>
</tr>
<tr>
<td>Attended RMIT University compulsory information sessions/animal ethics workshop?</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.*

List the procedures this person will be doing in this project and their level of experience with this species

<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience (Approx. no. of times procedure performed with this species)</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Intranasal administration of agents</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Oral gavage</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Intravenous administration of agents</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Intraperitoneal injection of agents</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
<tr>
<td>Pair feeding</td>
<td>Mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
</tr>
</tbody>
</table>

**Personnel Details**

<table>
<thead>
<tr>
<th>Please specify role:</th>
<th>Principal Investigator</th>
<th>Co-Investigator</th>
<th>Other Staff (indicate):</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Title/Full Name</th>
<th>A/Prof Steven Bozinovski</th>
</tr>
</thead>
<tbody>
<tr>
<td>Email</td>
<td></td>
</tr>
<tr>
<td>Qualifications</td>
<td>PhD, University of Melbourne; Bachelor of Science (Honours), La Trobe University</td>
</tr>
<tr>
<td>Position</td>
<td>Associate Professor</td>
</tr>
</tbody>
</table>

Page 25 of 35
RMIT University Animal Ethics Committee
Application Form for the Use of Animals for Scientific Purposes in Research & Teaching

Department/Institution: School of Health Sciences  
Scientific Licence*: SPPL365

Attended RMIT University compulsory information sessions/animal ethics workshop?  
[ ] Yes  [ ] No  [ ] NA

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species

<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>Mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal treatment</td>
<td>Mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal injection of agents</td>
<td>Mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>Mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>Intranasal administration of agents</td>
<td>Mouse</td>
<td>☑</td>
<td></td>
</tr>
</tbody>
</table>

Add row  Remove row

Personnel Details

Please specify role:  
[ ] Principal Investigator  [ ] Co-Investigator  [ ] Other Staff (indicate): Research Fellow

Title/Full Name: Dr Samantha Louise Passey  
Staff ID: [Redacted]

Email: [Redacted]  
Student ID: [Redacted]

Qualifications: PhD Biochemistry, BSc. (Honours) Biochemistry, University of Bristol.  
Position: Research Fellow

Department/Institution: School of Health Sciences  
Scientific Licence*: SPPL365

Attended RMIT University compulsory information sessions/animal ethics workshop?  
[ ] Yes  [ ] No  [ ] NA

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species
<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for</td>
<td>mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>intranasal administration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrapерitoneal injection</td>
<td>mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via</td>
<td>mouse</td>
<td>☑</td>
<td></td>
</tr>
<tr>
<td>intraperitoneal injection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral gavage</td>
<td>mouse</td>
<td>☑</td>
<td>A/Prof Ross Vlahos, School of Health Sciences, RMIT</td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>mouse</td>
<td>☑</td>
<td>A/Prof Ross Vlahos, School of Health Sciences, RMIT</td>
</tr>
<tr>
<td>Intravenous administration</td>
<td>mouse</td>
<td>☑</td>
<td>A/Prof Ross Vlahos, School of Health Sciences, RMIT</td>
</tr>
<tr>
<td>Pair feeding</td>
<td>mouse</td>
<td>☑</td>
<td></td>
</tr>
</tbody>
</table>

**Personnel Details**

Please specify role:  ☑ Principal Investigator  ☑ Co-Investigator  ☑ Other Staff (indicate):  ☑ Research Assistant

<table>
<thead>
<tr>
<th>Title/Full Name</th>
<th>Mr Huei Jiunn Seow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Email</td>
<td><a href="mailto:xxxxxxxx@gmail.com">xxxxxxxx@gmail.com</a></td>
</tr>
<tr>
<td>Qualifications</td>
<td>BSc honours, BBiomedSc</td>
</tr>
<tr>
<td>Department/Institution</td>
<td>School of Health Sciences</td>
</tr>
<tr>
<td>Scientific Licence*</td>
<td>SPPL365</td>
</tr>
<tr>
<td>Attended RMIT University compulsory information sessions/animal ethics workshop?</td>
<td>☑ No  ☑ Yes  ☑ NA</td>
</tr>
</tbody>
</table>

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species
<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>None (&lt;5)</th>
<th>Limited (5-20)</th>
<th>High (&gt;20)</th>
<th>If Level of Experience =&lt;20, indicate who will provide training and provide details below (e.g., will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal administration</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal injection</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Oral gavage</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>mouse</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>A/Prof Ross Vlahos, School of Health Sciences, RMIT</td>
</tr>
<tr>
<td>Intravenous administration</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Pair feeding</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

**Add row**  **Remove row**

**Personnel Details**

Please specify role:  
- [  ] Principal Investigator  
- [  ] Co-Investigator  
- [  ] Other Staff (indicate):  
  - Masters Student

Title/Full Name: Mr Ivan Bernardo  
Email: [ ]  
Qualifications: BSc  
Department/Institution: School of Health Sciences  
Scientific Licence*: SPPL365  
Attended RMIT University compulsory information sessions/animal ethics workshop?  
- [  ] Yes  
- [  ] No  
- [  ] NA

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species
<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>None (&lt;5)</th>
<th>Limited (5-20)</th>
<th>High (&gt;20)</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal administration</td>
<td>mouse</td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td>A/Prof Ross Vlahos, School of Health Sciences</td>
</tr>
<tr>
<td>Intraperitoneal injection</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral gavage</td>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>mouse</td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td>A/Prof Ross Vlahos, School of Health Sciences</td>
</tr>
<tr>
<td>Intravenous administration</td>
<td>mouse</td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td>A/Prof Ross Vlahos, School of Health Sciences</td>
</tr>
<tr>
<td>Pair feeding</td>
<td>mouse</td>
<td>×</td>
<td></td>
<td></td>
<td></td>
<td>Dr Samantha Passey, School of Health Sciences</td>
</tr>
</tbody>
</table>

**Personnel Details**

Please specify role:  
- [ ] Principal Investigator  
- [ ] Co-Investigator  
- [X] Other Staff (indicate): Research Assistant

Title/Full Name: Mr Selcuk Yatmaz

Email: [hiden]

Qualifications: BBioMedSc, BSc (honours)

Department/Institution: School of Health Sciences

Scientific Licence*: SPPL365

Attended RMIT University compulsory information sessions/animal ethics workshop?  
- [X] Yes  
- [ ] No  
- [ ] NA

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species.
<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience (Approx. no. of times procedure performed with this species)</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal administration</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal injection</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Oral gavage</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Intravenous administration</td>
<td>mouse</td>
<td>☒</td>
<td></td>
</tr>
<tr>
<td>Pair feeding</td>
<td>mouse</td>
<td>☒</td>
<td>Dr Samantha Passey, School of Health Sciences</td>
</tr>
</tbody>
</table>

**Personnel Details**

- Title/Full Name: Miss Victoria Austin
- Email: [Email]
- Qualifications: BBiomed (Honours)
- Department/Institution: School of Health Sciences
- Scientific Licence*: SPPL365
- Attended RMIT University compulsory information sessions/animal ethics workshop?: ☒ Yes, ☐ No, ☐ NA

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species
<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expose mice to cigarette smoke</td>
<td>mouse</td>
<td>(Approx. no. of times procedure performed with this species)</td>
<td>None (&lt;=5)</td>
</tr>
<tr>
<td>Methoxyflurane anaesthesia for intranasal administration</td>
<td>mouse</td>
<td></td>
<td>❌</td>
</tr>
<tr>
<td>Intraperitoneal injection</td>
<td>mouse</td>
<td></td>
<td>❌</td>
</tr>
<tr>
<td>Anaesthesia overdose via intraperitoneal injection</td>
<td>mouse</td>
<td></td>
<td>❌</td>
</tr>
<tr>
<td>Oral gavage</td>
<td>mouse</td>
<td>❌</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>mouse</td>
<td>❌</td>
<td></td>
</tr>
<tr>
<td>Intravenous administration</td>
<td>mouse</td>
<td>❌</td>
<td></td>
</tr>
<tr>
<td>Pair feeding</td>
<td>mouse</td>
<td>❌</td>
<td></td>
</tr>
</tbody>
</table>

**Personnel Details**

Please specify role:  
- [ ] Principal Investigator  
- [ ] Co-Investigator  
- [x] Other Staff (indicate): RAF staff

Title/Full Name: Tricia Murphy  
Email: [removed]  
Qualifications: Animal Facility Coordinator  
Department/Institution: RMIT Animal Facility  
Scientific Licence*: Yes  

Attended RMIT University compulsory information sessions/animal ethics workshop?  
- [x] Yes  
- [ ] No  
- [ ] NA

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species

<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear-tagging</td>
<td>mouse</td>
<td>(Approx. no. of times procedure performed with this species)</td>
<td>None (&lt;=5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>❌</td>
</tr>
</tbody>
</table>

**Personnel Details**

Please specify role:  
- [ ] Principal Investigator  
- [ ] Co-Investigator  
- [x] Other Staff (indicate): RAF staff

Title/Full Name: Joo Lee Cham  
Email: [removed]
Qualifications: 
Position: breeding and quarantine facility officer

Department/Institution: RMIT Animal Facility 
Scientific Licence:

Attended RMIT University compulsory information sessions/animal ethics workshop? 
- Yes ☒ 
- No   
- NA   

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species

<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>(Approx. no. of times procedure performed with this species)</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear-tagging</td>
<td>mouse</td>
<td>High (&gt;20)</td>
<td>☒</td>
<td></td>
</tr>
</tbody>
</table>

Personnel Details

Please specify role: 
- Principal Investigator
- Co-Investigator 
- Other Staff (indicate): Animal facility assistant 

Title/Full Name: Emma Collinson 
Staff ID
Student ID

Qualifications: Position: Animal facility assistant 

Department/Institution: RMIT Animal Facility 
Scientific Licence:

Attended RMIT University compulsory information sessions/animal ethics workshop? 
- Yes ☒ 
- No   
- NA   

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species

<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>(Approx. no. of times procedure performed with this species)</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear-tagging</td>
<td>Mouse</td>
<td>High (&gt;20)</td>
<td>☒</td>
<td></td>
</tr>
</tbody>
</table>

Personnel Details

Please specify role: 
- Principal Investigator
- Co-Investigator 
- Other Staff (indicate): Animal facility assistant 

Title/Full Name: Vikki Smyth 
Staff ID
Student ID

Qualifications: Position: Animal facility assistant 

Department/Institution: RMIT Animal Facility 
Scientific Licence:

Attended RMIT University compulsory information sessions/animal ethics workshop? 
- Yes ☒ 
- No   
- NA   

*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.
*Non-RMIT collaborators are assumed to be working under the authorisation of their “home” Scientific License, unless the clause below stating otherwise is completed by the Scientific Licence Nominee.

List the procedures this person will be doing in this project and their level of experience with this species

<table>
<thead>
<tr>
<th>Technique/Procedure</th>
<th>Species</th>
<th>Level of Experience</th>
<th>(Approx. no. of times procedure performed with this species)</th>
<th>If Level of Experience &lt;=20, indicate who will provide training and provide details below (e.g. will the Animal Welfare Officer provide training?)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear-tagging</td>
<td>mouse</td>
<td>None (&lt;5)</td>
<td>Limited (5-20)</td>
<td>High (&gt;20)</td>
</tr>
</tbody>
</table>

Add row | Remove row

Add Personnel | Remove Personnel
DECLARATION

By submitting this application, **I, the Principal Investigator** declare that:

- I have the appropriate qualifications and experience to perform the procedures described in this Application or to ensure that they are done correctly;
- I have read the provisions of Part IV of the *Prevention of Cruelty to Animals Act 1986/Regulations 2008*, including Amendments, (the Act) and the current version of the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (the Code);
- I understand that scientific/teaching activities involving the use of animals must NOT begin prior to receiving written approval from the RMIT University AEC;
- I accept responsibility for the conduct of the experimental procedures detailed above, in accordance with the requirements of the Act and the Code and any other conditions specified by the RMIT University AEC;
- I understand that I am required to promptly supply annual and final reports as a condition of approval;
- I further declare that the procedures described in this Application do not constitute unnecessary repetition of work previously carried out by other researchers or myself (Section 1.11 of the Code);
- I declare that all persons engaged in this project have been adequately instructed in, and are competent to perform, procedures they are to carry out. If they are not already skilled in the procedures, I will be responsible for ensuring that they obtain the necessary training, so that each procedure on an animal will be carried out in the most appropriate manner;
- I declare that all persons engaged in this project have copies / or access to copies of this Application form and any relevant standard operating procedures;
- I acknowledge and declare that I am responsible for ensuring that all regulated approvals, permits and clearances have been obtained prior to undertaking any research described in this Application;
- I have discussed this project with the Animal Facilities Manager who has indicated that the required animals can be maintained in the animal facility subject to space availability;
- I consent for my name and this Application document to be provided to, and kept on file with this AEC;
- I have ensured that the head of school has sighted this application and that s/he agrees that the required academic expertise and resources are available to complete this proposed research. Evidence of this assurance will be retained.

By submitting this application, **we, the Principal Investigator and Co-Investigators**, declare that we:

- have the appropriate qualifications and experience or will be trained by the person specified to perform the procedures as described in this application;
- have read the above application;
- have read the provisions of Part III of the *Prevention of Cruelty to Animals Act 1986* (including Amendments) and the latest version of the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*;
- agree to submit to the authority of the Principal Investigator, the Head of Faculty/School /Department and Scientific Licence Nominee listed in the section below for the purposes of collaboration on this project;
- consent for our names and this Application document to be provided to RMIT University AEC and Animal Ethics Office, and kept on file and in a confidential database. We understand that this information may be used in reports made to the University or any government department with legal authority to access this information.

---

1. The application must be submitted electronically by the Chief Investigator from his/her RMIT staff email account.

2. This evidence may consist of a hard-copy signed document or an email from the co-investigator agreeing to participate as described in the application.
CHECKLIST FOR APPLICANTS

Please complete:

**Project Title**
Does it describe the work proposed? [X] Yes  [ ] No

**Project Duration**
Is the proposed duration stated? [X] Yes  [ ] No

**Safety**
Does it describe the work proposed? [X] Yes  [ ] No

**Justification for the use of animals**
Is the project summary easily understood by people who do not have a scientific background? [X] Yes  [ ] No
Are the aims clearly stated? [X] Yes  [ ] No
Is the significance of the work clear? [X] Yes  [ ] No
Broadly what is going to be done to the animals? [X] Yes  [ ] No

**Replacement**
Is it clear why alternatives are not being used? [X] Yes  [ ] No

**Reduction**
Are the numbers requested justified? [X] Yes  [ ] No

**Refinement**
**Do I know...**
The meaning of all terms used? [X] Yes  [ ] No
Where will the animals be housed and who will care for them at all stages of the project? [X] Yes  [ ] No
Whether any genetically modified animals have phenotypes which require special care? [X] Yes  [ ] No
The details of what will happen to each individual animal or group of animals from the beginning to the end of the project? (agents, dose rates, routes and frequency of administration, actions, anaesthesia, surgery, number of procedures per animal, etc.). [X] Yes  [ ] No
The potential impact on the animals’ welfare of each procedure? [X] Yes  [ ] No
What criteria will be used to monitor the animals? [X] Yes  [ ] No
What will be done if welfare problems are identified? [X] Yes  [ ] No
How the animals will be killed and disposed of? [X] Yes  [ ] No
Whether early and subsequent inspections of the work are needed? [X] Yes  [ ] No

**Investigators**
**Do I know...**
Who will be doing the work? [X] Yes  [ ] No
What experience the named personnel have in the specific techniques described in the proposal? [X] Yes  [ ] No
What training is needed? [X] Yes  [ ] No
Who will provide the training? [X] Yes  [ ] No
How the training will be provided? [X] Yes  [ ] No
Have I consulted with the Animal Welfare Officer in the design of this project? [X] Yes  [ ] No
Provide an explanation for the number of animals being requested. This explanation should be based on statistical analysis and/or other considerations in the experimental design (e.g., multiple time points, quantity of tissue required). Where appropriate, present the numbers in table form.

The group size of 12 per group (8 [biochemistry] + 4 [histology]) has been based on power calculations to ensure a maximum likelihood of detecting real differences in parameters between control and treated mice based on historical variances in our laboratory for similar protocols (University of Melbourne AEEC #02084, 02085, 05097, 05107, 06199, 0810912, 1011596, 1112185). The experiments have been designed to extract the maximum information from as few mice as possible. While the animal numbers listed below seem large, each experiment has been designed to produce definitive outcomes preventing possible repeats of experiments.

**Experiment outline**

In the tables below we have outlined the experimental approach and the two main analyses required for our work which include the assessment of the lung by either biochemical analysis or via histopathology. Therefore our experimental endpoints require using separate animal groups due to the incompatibility of the techniques used. Animals used for histopathology cannot be bronchoalveolar lavaged (BAL) to prevent damage to the delicate alveolar and other lung structures. In addition the fixative used to preserve the lung tissue in the histopathological process prevents the assessment of genes and other cytokines due to tissue processing.

**Analyses 1: Determination of inflammation and COPD in the lung by biochemical analyses.**

One important aspect to inflammatory lung disease, COPD and AE-COPD is the profile of inflammatory cells which enter the pulmonary system. In order to assess whether the potentially therapeutic agents are effective, we look at the BAL to evaluate cytokine levels and cell profiles. Furthermore, the lungs from the same mice are tested for gene expression and for protein content on various disease mediators. We are therefore unable to assess lung architecture from animals which are used for BAL, gene and cytokine assessment.

**Analyses 2: Determination of inflammation, airway space enlargement and COPD in the lung by histological assessment in tissue.**

For this technique we need separate animals from the biochemically assessed for histology to determine any structural changes that have occurred in response to cigarette smoke. These lungs cannot be used for the above analyses as the lungs are fixed with paraformaldehyde (fixative) and thus cannot be used for molecular profiling as the fixative destroys the genes of interest. In addition, lungs that have been lavaged cannot be used for histological analyses because the pressures produced by the lavage process disrupt the lung structure. Therefore, two separate analyses (and hence groups of animals) are required for our experiments due to the incompatibility of the techniques used. This incompatibility in techniques does not allow both analyses to be performed on the same animal. Although the numbers required appear large, each experiment has been designed to produce definitive outcomes such that there is no need to go back and repeat experiments and we therefore in fact minimise the number of animals used.
Table 1: Smoke exposure protocol
Cigarette smoke exposure will be for 4 days (acute smoke protocol) or up to 6 months (chronic smoke protocol) as established from our previous work (AEEC # 02085, 05097, 0810912, 1112185).

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment</th>
<th>n</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c</td>
<td>Sham control</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Sham + Vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Sham + drug dose 3 (highest</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td>dose in the smoke animals)</td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Smoke control</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Smoke + Vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Smoke + drug dose 1</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Smoke + drug dose 2</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Smoke + drug dose 3</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Pair fed to smoke control</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Pair fed to smoke vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Pair fed to smoke drug dose 3</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>Sub-total</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>

The 12 animals per group will be divided into two subsections
8 animals per group for biochemistry and cell assessment
4 animals per group for histopathological assessment

However this will be done with 7 agents so 132 x 7 = 924 mice total
Agents will be tested in both acute (4-day) and chronic (up to 6 month) protocols, therefore 2 timepoints.
924 mice x 2 = 1848 mice

*Agents = small molecular weight compounds or neutralising antibodies
Table 2: Smoke exposure protocol in gene-modified mice

Based on our past research, we have acquired considerable evidence implicating oxidative stress enzymes (Gpx-1, NOX-1, NOX-2, NOX-4), and cytokines (IL-17) in COPD. We now wish to test the universality of oxidative stress enzymes and the cytokines by exposing mice lacking these genes to cigarette smoke.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment</th>
<th>n</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type C57Bl/6</td>
<td>sham</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Sham + vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Sham + drug</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Smoke control</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Smoke + vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Smoke + drug</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Pair fed to smoke</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Pair fed to Smoke +</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td>vehicle</td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>Wild-type C57Bl/6</td>
<td>Pair fed to smoke +</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td>drug</td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>sham</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Sham + vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Sham + drug</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Smoke control</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Smoke + vehicle</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Smoke + drug</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Pair fed to smoke</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Pair fed to Smoke +</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td>vehicle</td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td>KO</td>
<td>Pair fed to smoke</td>
<td>12</td>
<td>8 x Cellular inflammation and gene profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x Histopathological assessment</td>
</tr>
<tr>
<td></td>
<td>4 x Histopathological assessment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>216</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These studies will be conducted in 5 different mouse strains genetically deficient in genes that we have identified as being involved in oxidative stress responses and the development of COPD.

Therefore the total number of mice for KO mouse studies = 216 x 5 = 1080
Section 5.3 – Mouse Monitoring Checklist

Principal Investigator: Associate Professor Ross Vlahos

Secondary contact person: Mr Huei Jinn Seow

Agreed frequency of monitoring:

Cage or animal number(s) covered by this sheet:

**Observations (Tick any that apply):**

<table>
<thead>
<tr>
<th>Common/Procedures performed/Actions taken (include date):</th>
<th>initials:</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Abnormal respiration</th>
<th>Abnormal response to provocation</th>
<th>Abnormal appearance (coat condition, body condition &amp; posture)</th>
<th>Abnormal behaviour (or activity levels)</th>
<th>Abnormal response to provocation</th>
<th>Date:</th>
<th>Experimental day #:</th>
<th>Time:</th>
<th>Animal no.:</th>
<th>Normal weight (g):</th>
<th>Abnormal weight loss? (g):</th>
<th>no. (s):</th>
<th>Initials:</th>
</tr>
</thead>
</table>

Comments/Procedures performed/Actions taken (include date):

Initials:

Date:
<table>
<thead>
<tr>
<th>Date:</th>
<th>Experimental day #:</th>
<th>Time:</th>
<th>Animal no.(s):</th>
<th>Body weight (g):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weight loss?</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal appearance (coat condition, body condition &amp; posture)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal behaviour (or activity levels)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal response to provocation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal respiration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Other signs</td>
</tr>
<tr>
<td>Comments/Procedures performed/Actions taken (include Fate):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initials:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Section 5.4 – Intervention Criteria

**Steps:**
1. Determine the severity of abnormalities seen, using the Severity Table below.
2. Contact the project supervisor or animal house staff for advice if appropriate or required.
3. Take action in accordance with the Intervention Criteria below.

### Intervention Criteria

**Criteria are based on the severity of signs, as classified by the table below:**

<table>
<thead>
<tr>
<th>Observations (based on the Severity Table below)</th>
<th>Action required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ((\checkmark) on monitoring sheet)</td>
<td>None</td>
</tr>
<tr>
<td>1 or more “mild to moderate” signs (based on below)</td>
<td>Increase frequency of observations to twice daily (seek AFM/AWO advice where appropriate so that treatment and care can be given)</td>
</tr>
<tr>
<td>1 or more “severe” signs (based on below)</td>
<td>Euthanasia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Severity Table for Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild to Moderate</td>
</tr>
</tbody>
</table>

**Non-specific sign(s):**

- **Appearance**
  - Slight to moderate piloerection with no dehydration (skin tenting).
  - Piloerection with dehydration (skin tenting).

- **Weight changes**
  - Slight to moderated weight decrease (<20% compared to starting weight for influenza-treated mice and <15% compared to starting weight for all other mice).
  - Weight decrease (>20% compared to starting weight for influenza-treated mice and >15% compared to starting weight for all other mice).

- **Behaviour**
  - Subdued but responsive, decreased interaction with peers.
  - Unresponsive to activity and provocation.

**Specific conditions or abnormal clinical sign(s):**

- **Malocclusion**
  - If no to minimal oral trauma & no to minimal weight loss, seek AFM/AWO advice: consider trimming & providing powdered diet.
  - If oral trauma & weight loss seen, or if not responsive to treatment, euthanize.

- **Skin lesion(s)**
  - If very superficial and <1cm, seek AFM/AWO advice: consider treating with topical or systemic antibiotics.
  - If deeper layers of skin are exposed, or if not responsive to treatment, euthanize.

- **Fight wound(s)**
  - If very superficial and <5mm, seek AFM/AWO advice: consider treating with topical or systemic antibiotics. Recheck compatibility with cagemates.
  - If deeper layers of skin are exposed, or if not responsive to treatment, euthanize.

- **Rectal or Vaginal prolapse**
  - If has occurred very recently and the tissue is not necrotic, seek AWO advice: replacement sometimes possible under anaesthesia.
  - If has occurred more than several hours ago or tissue is necrotic, euthanize.

- **Eye discharge or conjunctivitis**
  - If mild and no other signs, seek AWO advice re treatment.
  - If other signs noted, or not responsive to treatment, euthanize.

- **Swelling(s)**
  - If not ulcerated, no other signs noted and does not impair movement, observe.
  - If has occurred more than several hours ago or tissue is necrotic, euthanize.

- **Other signs**
  - Hunched appearance, laboured breathing, loss of grooming. Seek AFM/AWO advice re: appropriate action or euthanize if animal is in moderate or severe pain or distress.

AFM = Animal Facility Manager, AWO = Animal Welfare Officer
APPLICATION FOR APPROVAL TO AMEND AN APPROVED RESEARCH PROJECT

1. ADMINISTRATION DETAILS

<table>
<thead>
<tr>
<th>SPPL:</th>
<th>Physiology (031)</th>
<th>ETHICS ID:</th>
<th>1212675.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC:</td>
<td>Anatomy &amp; Neuroscience, Pathology, Pharmacology, and Physiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROJECT SUPERVISOR &amp; DEPARTMENT:</td>
<td>Prof Mary Wlodek Physiology</td>
<td>PRIMARY CONTACT &amp; DEPARTMENT:</td>
<td>Mr Andrew Jefferies Physiology</td>
</tr>
<tr>
<td>TITLE:</td>
<td>Impact Of Exercise Early In Life On The Adult Heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APPROVAL DATE:</td>
<td>07 October 2014</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2 AMENDMENT SUMMARY

'Yes' answers in this section indicate amendments have been made to that section of the following application:

**Project Overview - SPPL or Title?**

No

**Risk Management/Permits?**

No

**Project Summary - Outline, Aims and Design?**

*Explain briefly why an amendment to the project is need, then give details in Project Summary Steps.*

Yes - Addition of tail vein blood sampling at 9, 16, 20 & 24 weeks.

**JUSTIFICATION FOR THE USE OF ANIMALS**

**Potential Benefits or Impact?**

No

**Repeated Studies/Use of Animals?**

No

**The 3Rs - Replacement, Reduction, Refinement?**

*Any request for more animals or revisions to project design will require an answer to this section in addition to reconsideration of all remaining Steps under the category of Refinement, Animals Requested, Transport and Location, Project Description, Personnel, Monitoring and Fate of Animals.*

No

**Animals Requested?**

*State briefly the changes made and complete new details in Animals Requested Step. Note: Both original approval and amended animal numbers and details will appear in the amended application. Please attach a table listing accumulated totals of each species/strain used to date.*

Yes - We request an additional 55 male WKY rats.

N=20 to be killed at 9 weeks of age (N=10 exercised & N=10 sedentary)

N=30 to be killed at 24 weeks of age (N=10 sedentary, N=10 early exercise, N=10 late exercise)

We also request an additional 5 animals to account for animal ill health or equipment failure.

**Transport and Location?**

No

**Project Description?**

*Briefly explain how in this amendment the new experimental design or the change to animal numbers can be reconciled with the original approved application. Include full details at Project Description Step.*

Yes - Addition of tail vein blood sampling at 9, 16, 20 & 24 weeks.

Timeline for 9 week and 24 week animals for molecular and hormone studies.

**PERSONNEL?**

*List here names of any new person being added and/or existing person being removed and a brief reason for doing so.*
Then complete all changes in Personnel Step.

Yes - Delete Kristina Anevska, Isobelo Pre and Tina Hosseini. Addition of tail vein blood sampling for some staff and students.

Monitoring or Management of Emergencies?

No

Fate of the Animals?

No

Attachments or Supporting Documents?

What is being added or taken out and why? E.g., updated monitoring sheet which incorporates new procedures being proposed.

Yes - Addition of updated experimental timeline and amendment letter.

2. PROJECT SUMMARY

BACKGROUND, AIMS AND OUTLINE

2.1 Provide a brief discussion of the background of the project and describe the progress of the project to date.

Cardiovascular disease is the largest cause of mortality in Australia and physical activity is a key protective factor in the prevention of cardiovascular disease and all-cause mortality. Many studies in adult rats and adult humans have shown that endurance exercise training is beneficial to the heart, but these effects are only temporary and lost several weeks after training is stopped. Because of this it has generally been assumed that the beneficial effects of exercise for the heart are also temporary for children. However we have exciting preliminary data from a previous study in rodents (AEC 0006228) suggesting that when young rats do exercise training before they reach puberty, their hearts become bigger (physiological hypertrophy) and possibly healthier and this beneficial effect lasts well into adulthood. Furthermore, this beneficial effect appears permanent, despite these rats being sedentary throughout their adult life. This project will test our hypothesis, that the juvenile period in rats is a unique stage of development amenable to long-term cardiac programming by short-term endurance training. At this formative stage of research, it is not possible to identify such a novel link in the heart using human models. Very little is known in humans about the long-term beneficial effects of endurance training during childhood on adult health. Support for our hypothesis is found in the few human epidemiology studies on earlier childhood that have been undertaken -an example being that the most active children at 5 years of age have lower fat mass at 8 and 11 years of age, even if they are sedentary after 5 years of age. This human study and our preliminary rodent findings support a role for endurance training during early life as being beneficial to adult health -despite a sedentary adult lifestyle. Well controlled animal studies free from confounding environmental and social factors are now needed to establish this proof of principle prior to human translation. Therefore, using a rodent endurance-training model that is well established by our research team the aims of this project are listed below:

2.2 State the aims of the project.

Aim 1: To investigate if Early Exercise increases adult cardiac function, dimensions, capillarisation, protein synthesis, and cardiomyocyte size and number consistent with physiological cardiac hypertrophy.

A) Assessment of cardiac function (M-mode and colour guided Doppler echocardiography in lightly anesthetised rats)

B) Measurement of ventricular wall thickness and lumen area (digital imaging)

C) Stereological assessment of capillarisation within the myocardium (stereological techniques)
D) Assessment of cardiac fibrosis (image analysis)

E) Assessment of cardiomyocyte growth (confocal microscopy, stereological measurement of the number of cardiomyocyte nuclei)

**Aim 2:** To establish if the juvenile period in rats is a unique stage of cardiac development amenable to physiological cardiac hypertrophy into adulthood by short-term endurance training.

**Aim 3:** To examine if there are gender-specific effects of Early Exercise on adult heart mass, cardiac function and heart health.

2.3 **Briefly outline what will happen to the animals to be used in the amended protocols. It is important to note that this section is a summary only. Expanded detail of procedures on animals is required in the Project Description.**

**Housing:** Rats are obtained from the ARC in Perth and are housed in plastic cages of 3-4 rats/cage, 12hr light cycle, standard environmental conditions and unrestricted pellets and water.

**Exercise training protocol 6mo cohort:** Exercise training consists of running on a motorized treadmill 5 days/week for four weeks. The 4 week exercise training intervention will be completed at different ages throughout the animals life. Some rats will do no exercise training (Sedentary), some rats will complete the exercise training early in life (at 5-9 weeks of age), other rats after puberty (at 11-15 weeks old) or later in life (at 20-24 weeks old).

**Metabolism Cage protocol:** At 5.5 months all animals will undergo acclimatization to a metabolism cage over several days. The animals will have a final 24hr met cage measurement, where food and water intake will be measured and urine and faeces output recorded.

**Echocardiography:** At 6 months of age, all animals will be placed under anaesthetic and an echocardiographic (ECG) assessment will be made.

**Post mortem:** Following ECG assessment at 6 months of age all rats will be killed by an overdose of anaesthetic and a post mortem conducted.

**Exercise training protocol 9wk cohort:** Exercise training protocol consists of running on a motorized treadmill 5 days/week for four weeks. Half of the 9 week animals will enter this protocol, the remaining animals will do no exercise (Sedentary).

**Echocardiography 9wk cohort:** At 9wks of age all animal will be placed under anaesthetic and an echocardiographic assessment made.

**Post mortem 9wk cohort:** Following ECG assessment all rats will be killed by an overdose of anaesthetic and a post mortem conducted.

**Tail vein blood sampling:** A tail vein blood sample will be obtained at 9, 16, 20 & 24 weeks to use for microRNA and hormones.

**JUSTIFICATION FOR THE USE OF ANIMALS**

2.4 **Explain the significance and the potential benefit of the proposed project.**

The innovation of this project is that it will establish that short-term endurance exercise during early life can permanently program improved adult heart health in rats. The significance of this project for human health is that should our findings translate into humans, it would mean that regular physical activity during childhood could result in improved adult heart health, even if people were sedentary during adulthood. Importantly, childhood represents a stage in life in which government intervention in physical activity levels is more feasible than during adulthood. For example, the amount and type of physical activity could be mandated by changes to the school curriculum. The outcomes from this proof of principle project would mean that short-term, practical public health interventions in childhood could improve adult heart health and dramatically reduce the burden of disease on the Australian health system.

2.5 **Will the degree of pain or distress experienced by animals be the same as outlined in the original project.**

The echocardiography of the heart occurs under anaesthetic. There are no ill effects of the echo probe procedure. The animals will be killed by anaesthetic overdose following the ECG procedure.

We have used this exercise training protocol in previous rodent exercise studies (0006228). Based on our previous experience, rats cope well with treadmill running exercises such as those proposed in the present study as they are naturally physically active animals. Some rats require encouragement such as gentle tapping or blowing some medium pressure air onto their "backsides" in order for them to complete the exercise procedure.
This could be interpreted as a mild form of discomfort, similar to what humans feel when they are exercising.

Blood will be sampled directly from the tail vein. This involves gently warming the rat using a temperature controlled heat lamp. The rat is exposed to heat for no longer than 10 minutes at a cage temperature of 35-37°C. During this time the rat is visually monitored to ensure that the temperature is not causing discomfort or considerable stress. The rats cope well with heating, and usually exhibit no distress. Rats are then wrapped gently and blood is sampled from the caudal vein. The rats remains wrapped for approximately 1 minute for the tail vein blood sample. The rats will flinch and often squeal when the needle is inserted, to ensure sample is done efficiently they are gentle restrained. Once the sample is taken the rat is unwrapped and returns to normal behaviour. Staff have extensive experience in this technique to ensure that the stress caused and time taken is minimal. The pain caused to the rat is similar to that experienced when humans have a needle inserted for blood sampling. Rats are returned to the box and monitored for normal behaviour and to ensure the tail is not bleeding.

2.6 Does this project duplicate work that has been carried out previously? If so, please explain why it is necessary to duplicate the work.

No

2.7 Have any of the animals been the subject of a previous research or teaching activity? If yes, provide AEC Register Number/s of the other project/s, describe what was done to the animals previously, and justify their use in this project.

No

REPLACEMENT

3. ALTERNATIVES

3.1 Have alternatives that totally or partially replace the use of animals been incorporated into this project? If no, identify potential alternatives and explain why they are unsuitable for use in this project. If yes, please describe what alternatives are to be used in this project.

No - This project considers the effects of exercise early in life on the adult heart. There is no alternative to the use of the whole animal.

REDUCTION

4. ANIMALS REQUESTED

<table>
<thead>
<tr>
<th>APPROVED NUMBER OF ANIMALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type/Species</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Rat</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AMENDED NUMBER OF ANIMALS REQUESTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type/Species</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Rat</td>
</tr>
</tbody>
</table>

NOTE: refer to section 6.2 for details of genetically modified and/or cloned animals
5. JUSTIFICATION FOR NUMBER OF ANIMALS REQUESTED

5.1 Provide an explanation for the number of animals being requested. This explanation should be based on statistical analysis and/or other considerations in the experimental design (e.g., multiple time points, quantity of tissue required). Where appropriate, present the numbers in table form.

Please refer to Appendix one for description of animals required.

5.2 To reduce animal use, would the animals or their tissues be suitable for use in another project at the end of your experiment/s? Identify the suitable project, if known.

Yes - We will be taking a complete range of tissues at post mortem from these animals. We have strong collaborations both nationally and internationally and will be further examining if the tissues from this study which we ourselves do not use could form the basis of further study for collaborators.

REFINEMENT

6. USE OF ANIMALS

6.1 Justify your choice of animal (species/strain/sex/age).

The Wistar Kyoto rat strain is used as it is the same strain that we have used in our previous studies where we have established our preliminary findings. Therefore it is critical that the study be conducted in the same strain of rats. Mice are also commonly used in exercise experiments; however the tissue available from each mouse is much less. Therefore, by using rats instead of mice, fewer animals are needed to be killed in order to perform all of the planned analyses.

6.2 Genetic Modification or Cloning of Animals.

This project does not involve the use or production of genetically modified animals.

This project does not involve the use or production of cloned animals.

6.3 Transport

6.3.1 Will animals need to be transported from the source location/s to the location where they will be held for this project?

No

6.3.2 Where will procedures be performed? If animals need to be transported from where they are housed to where the procedures are carried out, provide details of transport and acclimatisation procedures.

ECG assessment will be performed within the Biological Research Facility. The animals will be transported in appropriate transport boxes containing their own bedding in the morning of the day of post mortem to the research laboratory on Level 6 of the Department of Physiology. They will be euthanized in this laboratory.

6.4 Location & Housing

6.4.1 Where will animals be housed?
### 6.4.2 Will any animals be housed outdoors? If, contrary to the needs of the species, no shelter is provided, justify the lack of shelter.

No

### 6.4.3 What type of housing will be used? Describe any special housing requirements.

Rats will be housed in plastic cages containing sawdust. They have constant access to water and food. Shredded paper and environmental enrichments are also provided for rats to prepare nests with.

### 6.4.4 Will any animals be housed individually? If yes, explain why, for how long and how the impact of social isolation will be minimised.

Yes - They will have a 24 hour metabolic measurement in which they are housed individually in a metabolic cage. This is necessary to ensure accurate measurement of food and water intake and energy expenditure during this experiment. This will occur on two separate occasions 3 days apart when they are 5 months old.

---

### 7. PROJECT DESCRIPTION

**Housing and food:** Rats will be obtained from the ARC and housed in the BRF. They will be kept in plastic cages of 3-4. The rats are exposed to a 12 hour light/dark cycle at a room temperature of 18-22 degrees C and 50% relative humidity. All rats have food available ad libitum as mouse cubes and unrestricted drinking water access. All rats have nesting materials available and enhancement items.

**Measurement of body weight and dimensions (AEC 1011865, 1112130):** Measurement of weight and dimensions (crown rump length, head width, limb length and abdominal circumference) 2, 4, and 6 months. Measurement of dimensions requires short animal restraint (30 seconds) and has no adverse effects on the rat.

**Exercise Training:** The exercise training protocol consists of running on a motorized treadmill 5 days/week. To allow for improvements in fitness and familiarity with the procedure, the running duration progressively increases in 10 minute increments each day from 20 to 60 minutes, with speed set at 15m/min for the first week and 20m/min thereafter. The Sedentary rats are placed on a stationary treadmill for an identical amount of time during weeks 5-9 of life. This is the same exercise training protocol as we have employed in our previous study (0006228). Although voluntary exercise with the use of running wheels is an alternative exercise option, the precise control of exercise intensity is only possible with treadmill exercise and the low variation within the groups allows for our physiologically meaningful changes to be detected. Time line for exercise training: There are 4 treatment groups of rats, with all rats being killed at 6 months of age. Treatment group 1 (Sedentary) are sedentary and are not subjected to any endurance training. Treatment group 2 (Early Exercise) complete the exercise training protocol during weeks 5-9 of age. Treatment group 3 (Mid Exercise) complete the exercise training protocol at weeks 11-15 of age. Treatment group 4 complete the exercise training protocol at weeks 20-24 of age. We will also study a cohort of 9wk rats. These animals will enter the exercise program at 5wks of age, and will exercise for 4 weeks using the same speed and duration of exercise as the early exercise group of the 6mo cohort. Only half of these 9wk animals will exercise, the remaining animals will have no exercise (sedantary).

**Renal function testing (AEC 1011865, 1112130):** At approximately 5.5 months rats undergo renal function testing using a metabolism cage. Prior to a 24hr measurement in the cage, the rats are acclimatised to the metabolism cage over 11 days. Day 1- 3hrs in metabolism cage, day 4- 8hrs, day 7- overnight, at day 11 the rats have a final 24hr measure.

**Echocardiography (AEC 1011865, 1112130) (Aim 1):** The assessment of left ventricular function will be assessed echocardiographically, using a GE Vivid E9 and rat cardiac probe at 6 months and 9 weeks of age. Examination requires a short period of isoflurane anaesthesia (approx 30 mins) using the Univentor 400 anaesthetic unit, using an induction concentration of 3-5% oxygen, at a flow-rate of 3L min, followed by a maintenance concentration of 1.5-3% (isoflurane) delivered by a head funnel. The animal will then be placed supinely onto a warming pad, and the chest shaved to remove fur. Ultrasound imaging is obtained with gentle application of the probe onto the chest wall, with medical ultrasound gel used as a coupling fluid. Measurement of left ventricular fractional shortening, ejection fraction and wall thickness will be estimated.
made.

**Post mortem:** Following ECG assessment rats will be killed with an overdose of an anaesthetic administered intraperitoneally (Ketamine (300mg/kg) and Illium Xylazil-20 (30mg/kg) causing a loss of consciousness and subsequent collection of blood and tissues for further analysis.

**Tail Vein Blood Sampling:** Blood will be sampled directly from the tail vein. This involves gently warming the rat using a temperature controlled heat lamp where the temperature can not increase above 38C. We use gentle heat for a longer period so as to minimise the stress to the animal during this procedure. The rat is exposed to heat for no longer than 10 minutes at a cage temperature of 35-37C. During this time the rat is visually monitored to ensure that the temperature is not causing discomfort or considerable stress. Rats are then wrapped gently and blood is sampled from the caudal vein using a 27 gauge needle on a 1ml syringe. Maximum volume sampled is 400μl. The rat remains wrapped for approximately 1 minute for the tail vein blood sample. Staff have extensive experience in this technique to ensure that the stress caused and time taken is minimal. Rats are returned to the box and monitored for normal behaviour and to ensure the tail is not bleeding. Blood samples will be obtained at 9, 16, 20 and 24 weeks.

**Timeline for 9 weeks cohort (for molecular and hormone analysis):** Animals will arrive at the BRF at 4 weeks of age and be allocated to one of two groups; sedentary and exercise. Those animals allocated to the exercise group will commence treadmill exercise at 5 weeks of age using the protocol outlined above. The sedentary group will have no exercise. At 9 weeks all animals from this cohort will have blood sampled from the tail vein and then be killed and tissues collected and weighed. Please see attached experimental timeline.

**Timeline for 24 weeks cohort (for molecular and hormone analysis):** Animals will arrive at the BRF at 4 weeks of age and be allocated to one of three groups; sedentary, early exercise or late exercise. Animals for the early exercise group will commence treadmill exercise at 5 weeks using the above protocol. Animals allocated to the late exercise group will commence treadmill exercise at 20 weeks using the above protocol. All animals allocated to this group will have blood sampled at 9, 16, 20 and 24 weeks of age via the tail vein. At 24 weeks of age all animals will be killed and tissues collected and weighed. Please see attached experimental timeline.

### 8. PERSONNEL

<table>
<thead>
<tr>
<th>PROJECT SUPERVISOR</th>
<th>Name</th>
<th>Qualifications</th>
<th>Department</th>
<th>Species</th>
<th>Technique /Procedure</th>
<th>Times Performed</th>
<th>Training Required</th>
<th>Training Provided by</th>
<th>Expertise of Trainer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wlodek, Prof Mary</td>
<td>Bachelors Degree, University of Western Ontario, PhD, Monash University, Masters (Research), University of Western Ontario</td>
<td>Physiology</td>
<td>Rat</td>
<td>Tail vein blood sampling</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jefferies, Mr Andrew</td>
<td>Bachelors Degree, La Trobe University, Post Graduate Diploma, La Trobe University</td>
<td>Physiology</td>
<td>Rat</td>
<td>Monitoring of rats for health and well being</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Intraperitoneal Injection for euthanasia</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Weights and dimensions</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Euthanasia by CO2 asphyxiation</td>
<td>more than 20</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Treadmill exercising of rats</td>
<td>0</td>
<td>Yes</td>
<td>Dr Glenn Wadley</td>
<td>Dr Wadley has over 10 years experience with rodent exercise experiments and has performed the procedure in</td>
</tr>
</tbody>
</table>
over 200 rats and mice.

<table>
<thead>
<tr>
<th>Name</th>
<th>Wadley, Dr Glenn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Dr Wadley is Senior Lecturer at Deakin University in Burwood. He has over 10 years experience in using the motorized treadmill to exercise rats. He will be responsible for teaching staff and students in the Wlodek laboratory the rat exercise protocol.</td>
</tr>
<tr>
<td>Department</td>
<td>Deakin University</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
</tr>
<tr>
<td>Rat</td>
<td>Treadmill exercising of rats</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Asif, Yasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td></td>
</tr>
<tr>
<td>Department</td>
<td>Deakin University</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
</tr>
<tr>
<td>Rat</td>
<td>Tail vein blood sampling</td>
</tr>
<tr>
<td>Rat</td>
<td>Monitoring of rats for health and well being</td>
</tr>
<tr>
<td>Rat</td>
<td>Intraperitoneal Injection for euthanasia</td>
</tr>
<tr>
<td>Rat</td>
<td>Treadmill exercising of rats</td>
</tr>
<tr>
<td>Rat</td>
<td>Weights and dimensions</td>
</tr>
<tr>
<td>Rat</td>
<td>Euthanasia by CO2 asphyxiation</td>
</tr>
<tr>
<td>Rat</td>
<td>echocardiography of heart</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Mahizir, Nurul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Nurul has completed her BSc with honours in 2012, and intends to commence her PhD in 2013 within the Wlodek Laboratory.</td>
</tr>
<tr>
<td>Department</td>
<td>Physiology</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
</tr>
<tr>
<td>Rat</td>
<td>Tail vein blood sampling</td>
</tr>
<tr>
<td>Rat</td>
<td>Monitoring of rats for health and well being</td>
</tr>
<tr>
<td>Rat</td>
<td>Intraperitoneal Injection for euthanasia</td>
</tr>
<tr>
<td>Rat</td>
<td>Euthanasia by CO2 asphyxiation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Cheong, Jean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualifications</td>
<td>Jean completed her BSc with honours in 2012, and intends on commencing her PhD in 2013 within the Wlodek Laboratory.</td>
</tr>
<tr>
<td>Department</td>
<td>Physiology</td>
</tr>
<tr>
<td>Species</td>
<td>Technique /Procedure</td>
</tr>
<tr>
<td>Rat</td>
<td>Tail vein blood sampling</td>
</tr>
<tr>
<td>Rat</td>
<td>Monitoring of rats for health and well being</td>
</tr>
<tr>
<td>Rat</td>
<td>Intraperitoneal</td>
</tr>
</tbody>
</table>
9. MONITORING

9.1 Day-to-day monitoring during the project.

Who will monitor the animals on weekdays?

Jean Cheong, Mr Andrew Jefferies, Nurul Mahizir, Yasmin Asif

Who will monitor the animals after hours (incl. Weekends and holidays)?

Jean Cheong, Mr Andrew Jefferies, Nurul Mahizir, Yasmin Asif

9.2 Day-to-day monitoring during the project: What specific signs will be monitored and how frequently? Attach a copy of the monitoring checklist you will use to record these observations.

We will monitor the daily health of the animals with visual checks to ensure they are active and appear healthy (ie. Eyes clear, no secretions, smooth hair, normal behaviour). BRF staff also handle the rats at least twice weekly and are trained to observe and act on any inappropriate behaviour or activity of the rat. On weekends and public holidays the animals will be checked once daily by a member of the research team. In addition, rats are checked daily by the staff of the BRF facility and immediate contact is made with an investigator should any animals appear unwell. In addition, the staff of the BRF change bedding two times each week and visually check the rats. All staff and students that have not been trained will be fully supervised at all times by Andrew Jefferies. No staff or students will be rostered onto weekend duties until they are fully trained.

9.3 Monitoring during and after procedures/interventions: What specific signs will be monitored and how frequently? Attach a copy of the monitoring checklist you will use to record these observations.

Throughout all study times observations are made visually and any activity which seems to be non-typical is recorded on our animal record sheets and reported to senior staff for assessment. Monitoring sheets are attached for these procedures. Rats will be continuously monitored during all treadmill running procedures for changes in running style (indicating fatigue). Rats will also be withdrawn if they are unable or unwilling to complete the running tasks without a small amount of encouragement as detailed in the project description.

9.4 What clinical, behavioural or other signs will be used to indicate that intervention is needed to alleviate an animal's pain or suffering? What action will be taken if these indicators are reached?

The rats will be monitored for changes in body mass (i.e. loss of appetite) and general coat condition (lack of grooming). If any animal shows signs of distress (losing body mass, scruffy coat, no grooming, not feeding or drinking, very aggressive or withdrawn behaviour etc) the animal will be euthanised. In our experience this is very uncommon in our healthy cohort of rats. Animals will be weighed and monitored daily whilst exercising, and if an animal loses weight over 3 days or max > 15% weight loss the animal will be removed from the study. To approach with treatment is often not possible and would most likely further affect the study results, so euthanasia
is the most common approach.

9.5 Who is responsible for the management of emergencies?

Prof Mary Wlodek, Mr Andrew Jefferies

10. FATE OF ANIMALS

10.1 What will be the maximum period of time that an individual animal or group of animals will be used in this project?

Rats will be in the experimental protocol for up to 6 months.

10.2 What will happen to the animals at the completion of the project?

The animals are killed and relevant tissues collected.

10.3 If the animals are to be killed, how will this be done and by whom? Include information about agents, dose rates, method and route of administration and experience of personnel.

Animals for post mortem tissue collection are killed via intraperitoneal overdose of Ketamine (300mg/kg) and Ilum Xylazil-20 (30mg/kg) using a 27G needle and final volume of no more than 500ul. After the overdose of Ketamine and Xylazine a thoracotomy will be performed to ensure death. All staff and students are proficient in these techniques, or will be thoroughly taught.

10.4 What will be the method of disposal of dead animals?

Animals are wrapped in black plastic bags and placed in the freezer in the BRF on level 9 for appropriate later disposal.

11. ADDITIONAL DETAILS

11.1 Risk Management

11.1.1 Does the research involve procedures or agents that might pose a health risk to other animals and/or personnel? If Yes, please explain the risk and describe what precautions will be taken.

Yes - Aerrane (Isoflurane) is an anaesthetic that is not considered hazardous according to criteria of Worksafe Australia. Although not hazardous, we take the following precautions to in minimise risk to other animals and personnel; scavenger system to remove excess anaesthetic and PPE (lab coats, gloves and safety glasses).

11.1.2 Is the acquisition, holding, or use of the animals subject to any permit, law or regulation of the State or Commonwealth (e.g., OGTR, protected native or imported)? If yes, please specify permit numbers.

No

11.2 Glossary of Scientific Terms

<table>
<thead>
<tr>
<th>Scientific Term</th>
<th>Lay Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiomyocyte</td>
<td>Muscle cells that make up the cardiac muscle</td>
</tr>
</tbody>
</table>
11.3 **Attachments**

*The following attachments should accompany this application:*

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Attached Via Themis</th>
<th>Hard Copy Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extension to question in application</td>
<td>Animal request explanation</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Extension to question in application</td>
<td>Experimental Timeline</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Monitoring checklist proforma</td>
<td>Echo monitoring sheet</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Monitoring checklist proforma</td>
<td>Exercise monitoring sheet</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Extension to question in application</td>
<td>Amendment Letter</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Extension to question in application</td>
<td>Experimental Timeline</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

11.4 **Funding and Contracts**

11.4.1 **Identify the principal source of funding for this project (internal/external/commercial or private).**

*External funding agency*

11.4.2 **Is this project Commercial in Confidence?**

No

11.4.3 **Is this project covered by a research contract?**

No

12. **GENETIC MODIFICATION OR CLONING OF ANIMALS**

None Specified.

13. **AUTHORISATIONS**

The Research Office has verified that the following nominated personnel have signed off on this project application online through THEMIS on the given date/s:

<table>
<thead>
<tr>
<th>Role</th>
<th>Name</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Supervisor</td>
<td>Wlodek, Prof Mary</td>
<td>18 September 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Jefferies, Mr Andrew</td>
<td>16 September 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Mahizar, Nurul</td>
<td>16 September 2014</td>
</tr>
<tr>
<td>Investigator</td>
<td>Cheong, Jean</td>
<td>17 September 2014</td>
</tr>
<tr>
<td>Animal Facility Manager</td>
<td>Kesar, Ms Marica</td>
<td>18 September 2014</td>
</tr>
</tbody>
</table>

The Project Supervisor has acknowledged that all trainers listed on this application have the relevant expertise and that they have accepted responsibility to train their nominated researcher/s to be competent in the necessary procedures.
Contact details for this correspondence:
Emmanuel Christie
Senior Animal Ethics Officer (Research Ethics & Integrity)
Researcher, Innovation and Commercialisation, The University of Melbourne

Telephone: [redacted]
Email: [redacted]

In reply, please quote Ethics ID 1212675.5

ATTENTION: Dr Jessica Griffiths

The University of Melbourne

Dear Prof Mary Wlodek,

Ethics ID: 1212675.5

Title: impact of exercise early in life on the adult heart

Thank you for your response to queries raised by the Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee at a meeting held on 11 December 2012 to consider the above-named application.

On behalf of the Committee the Chair has approved the application. Please see overleaf, Summary Details for Approved Animal Ethics Project and Conditions of Approval.

Please do not hesitate to contact me if you have any queries.

Kind regards,

Emmanuel Christie
Senior Animal Ethics Officer
Summary Details for Approved Animal Ethics Project

TITLE: Impact of exercise early in life on the adult heart

ETHICS ID: 1212675.5

APPROVAL DATE: 11 March 2013

RESPONSIBLE AEC: Anatomy & Neuroscience, Pathology, Pharmacology, and Physiology Animal Ethics Committee

SCIENTIFIC PROCEDURES PREMISES LICENCE: SPPL 20395

EXPIRY DATE: 30 September 2016

PRIMARY CONTACT: Prof Mary Wlodek

PROJECT SUPERVISOR: Prof Mary Wlodek

OTHER INVESTIGATORS: Dr Glenn Wadley

Yasmin Asif

Nurul Mahizir

Jean Cheong

Ivan Bernardo

Animals Approved:

<table>
<thead>
<tr>
<th>Category</th>
<th>Type/Species</th>
<th>Breed/Strain</th>
<th>Sex</th>
<th>Age</th>
<th>Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory Animals</td>
<td>Rat</td>
<td>Wistar</td>
<td>M</td>
<td>4 weeks</td>
<td>145</td>
</tr>
<tr>
<td>Laboratory Animals</td>
<td>Rat</td>
<td>Wistar</td>
<td>F</td>
<td>4 weeks</td>
<td>90</td>
</tr>
</tbody>
</table>

Conditions of Approval

Any amendment proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until approval is granted.

The AEC must be notified of:
1. any adverse incidents involving animals used in the project and the steps taken in response
2. any changes to approved investigators
3. change in location(s) for using and/or holding animals other than those specified in the application.

A record of details of any animals used in the project must be retained.

All work must be conducted in accordance with the Prevention of Cruelty to Animals Act 1986 and associated Regulations, and the Australian code for the care and use of animals for scientific purposes.