DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of the MD (Res) degree by the University of London.
Chemoprevention

in a

Validated Rat Model

of

Oesophageal Adenocarcinoma

Andrew Hindmarsh

A thesis submitted to the University of London

for the degree of Doctor of Medicine
ABSTRACT

The UK has experienced an increase in the incidence of oesophageal adenocarcinoma (OAC) in recent years. The prognosis for patients with OAC remains poor with currently available treatments prompting a search for alternative ‘chemopreventive’ treatments that inhibit oesophageal carcinogenesis. Both non-steroidal anti-inflammatory drugs (NSAIDS) and flavonoids are associated with a significant risk reduction for developing OAC in epidemiological studies. The aim of this study was to validate Levrat’s surgical model of OAC in the rat, and assess the chemopreventive effects of the NSAID aspirin, and the flavonoid quercetin on the development of OAC in the validated rat model.

METHODS: Levrat’s model was validated in a time course experiment. Morphological and molecular events occurring in the distal oesophagus during disease progression were determined and compared to human disease. The effect of aspirin and quercetin on disease initiation and progression was determined by commencing treatment either before the onset of reflux, or 4-weeks afterwards. The incidence of Barrett’s oesophagus (BO) and OAC within each group was determined, along with methylation levels of the ESR-1, p16 and HPP1 gene promoter regions.

RESULTS: The morphological and molecular changes in the distal oesophagus of the rat model are broadly consistent with those reported in human disease.
The incidence of OAC was significantly lower in aspirin treated rats. A non-significant reduction in incidence of OAC was observed with quercetin treatment. Timing of treatment with regard to onset of reflux had no significant effect on OAC development in either treatment group. Neither treatment significantly effected methylation levels within the gene promoters examined.

CONCLUSION: Use of Levrat’s model as a model of human OAC seems justified. Aspirin inhibits development of oesophageal adenocarcinoma induced by reflux in this rat model. No additional reduction in cancer incidence is observed if treatment is commenced prior to inception of reflux disease.
ACKNOWLEDGEMENTS

This research project would not have been possible without the help and support of a myriad of people at both The Institute of Food Research (IFR) and the Norfolk and Norwich University Hospital.

To start with I would like to thank Edward Cheong for providing a running start to the project. His tireless globetrotting in the search for a surgical model of oesophageal adenocarcinoma enabled the trouble free establishment of this model in Norwich, and his patience as a trainer in the art of the 7/0 anastomosis was very well appreciated. This was complemented by the enormous help and input with animal husbandry provided by Simon Deakin and Val, to whom I am very grateful.

I am eternally grateful to Virginia Sams and Lazlo Igali for the long hours they spent looking down a microscope, and to all the post docs at the IFR for their help and advice with the molecular experiments. In particular I would like to thank Joanne Doleman and Liz Lund for being my day to day mentors in the laboratory, and Nigel Belshaw for his assistance with all things methylated. Their contribution to this study was invaluable.

Finally I would sincerely like to thank my supervisors Michael Rhodes, and Ian Johnson, and Jo Martin for their unswerving support, help and advice during this study which made all things possible.
INDEX

1. INTRODUCTION 14
   1.1 OESOPHAGEAL CANCER 14
      1.1.1 Incidence 14
      1.1.2 Risk Factors 14
      1.1.3 Pathogenesis 16
   1.2 MOLECULAR EVENTS IN DISEASE PROGRESSION 20
      1.2.1 Background 20
      1.2.2 Genetic Events 20
      1.2.3 Epigenetic Events 24
   1.3 MANAGEMENT 29
      1.3.1 Treatment of OAC 29
      1.3.2 Early Detection Initiatives 31
         1.3.2i Two Week Wait Scheme 31
         1.3.2ii Barrett’s Screening Program 32
   1.4 PREVENTION OF BARRETT’S RELATED OESOPHAGEAL
      ADENOCARCINOMA 34
      1.4.1 Introduction 34
      1.4.2 Modification Of Refluxate; The Response to Acid Reduction 34
         1.4.2i Histamine 2 (H2) Receptor Antagonists 34
         1.4.2ii Proton Pump Inhibitors (PPI) 36
      1.4.3 Anti-Reflux Surgery 41
      1.4.4 Alternative Interventions - Mucosal Ablation and Resection 45
   1.5 CHEMOPREVENTION 53
      1.5.1 NSAIDS 53
      1.5.2 COX 57
         1.5.2i Arachadonic Acid Metabolism 57
         1.5.2ii COX-2 and Cancer Pathogenesis 59
         1.5.2iii COX-2 Expression and Oesophageal Adenocarcinoma 60
         1.5.2iv COX-2 and Chemoprevention 61
1.5.2v Potential Problems with NSAIDS and Selective COX-2 Inhibitors 63
1.5.3 Diet and the Flavonoids 64
1.5.3i The Flavonoids and Quercetin 64
1.5.3ii Mechanism of Action 68

1.6 RAT MODELS OF OESOPHAGEAL CANCER 71
1.6.1 Historical Development 71
1.6.2 Iron Supplementation 75
1.6.3 Are Oesophageal Adenocarcinomas reflux induced? 76
1.6.4 Duodenal versus Gastric Reflux 78
1.6.5 Is the Rat Model a good model of Human Disease? 78
1.6.6 Unanswered Questions about the Rat Model 82
1.6.7 Selection of a Rat Model for this study 82

2. AIMS 84

3. METHODS 85
3.1 STUDY DESIGN – Validation Study 85
3.1.1 General 85
3.1.2 Histological Analysis 87
3.1.3 Molecular Analysis 87
3.1.3i Methylation Studies 87
3.1.3ii Global Gene Transcription 88

3.2 STUDY DESIGN – Intervention Study 89
3.2.1 General 89
3.2.1i Pre-initiation Group 89
3.2.1ii Long-term Group 90
3.2.2 Sample Size and Power Calculation 92
3.2.2i Aspirin 92
3.2.2ii Quercetin 92
3.2.3 Drug Dosing – Dose Selection 93
3.2.3i Aspirin 93
3.2.3ii Quercetin 93
3.2.3iii Drug Administration 94
3.2.3iv Drug Blood Level Estimation 94
3.2.4 Histological Analysis 94
3.2.5 Methylation Studies 94
3.3 RAT MODEL 96
3.3.1 General 96
3.3.2 Surgical Induction of Reflux Disease 96
3.3.2i Pre-operative Handling and Stress Reduction 96
3.3.2ii Anaesthetic 97
3.3.2iii Oesophagojejunostomy 98
3.3.3 Recovery 100
3.4 TISSUE HARVEST 103
3.5 HISTOLOGICAL ANALYSIS 106
3.6 CpG ISLAND METHYLATION ANALYSIS 107
3.6.1 DNA Extraction 107
3.6.1i Release of DNA from Tissues 107
3.6.1ii Binding of DNA to Column 108
3.6.1iii Washing the Column 108
3.6.1iv DNA Elution 108
3.6.2 Quantification and Quality Control of Extracted DNA 109
3.6.3 Bisulphide Modification 110
3.6.4 Purification of Bisulphide Modified DNA 110
3.6.5 ESR-1 111
3.6.5i PCR Amplification 111
3.6.5ii Quantitative MSP Reaction 111
3.6.6 p16 113
3.6.6i PCR Amplification 113
3.6.6ii Quantitative MSP Reaction 113
3.6.7 HPP1 114
3.6.7i PCR Amplification 114
3.6.7ii Quantitative MSP Reaction 114
3.7 GENE EXPRESSION – MICROARRAY STUDIES 115
3.7.1 Overview 115
<table>
<thead>
<tr>
<th>Section</th>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7.2</td>
<td>RNA Extraction</td>
<td>115</td>
</tr>
<tr>
<td>3.7.2i</td>
<td>General</td>
<td>115</td>
</tr>
<tr>
<td>3.7.2ii</td>
<td>Release of RNA from Tissues</td>
<td>116</td>
</tr>
<tr>
<td>3.7.2iii</td>
<td>Binding of RNA to Column</td>
<td>117</td>
</tr>
<tr>
<td>3.7.2iv</td>
<td>Washing the Column</td>
<td>117</td>
</tr>
<tr>
<td>3.7.2v</td>
<td>RNA Elution</td>
<td>117</td>
</tr>
<tr>
<td>3.7.3</td>
<td>Total RNA Quantification and Quality Control</td>
<td>118</td>
</tr>
<tr>
<td>3.7.3i</td>
<td>Preparation of Gel-Dye Mix</td>
<td>118</td>
</tr>
<tr>
<td>3.7.3ii</td>
<td>Preparation of Nano labchip</td>
<td>118</td>
</tr>
<tr>
<td>3.7.3iii</td>
<td>Running the Chip</td>
<td>119</td>
</tr>
<tr>
<td>3.7.4</td>
<td>RNA Preparation and Labelling</td>
<td>120</td>
</tr>
<tr>
<td>3.7.4i</td>
<td>General</td>
<td>120</td>
</tr>
<tr>
<td>3.7.4ii</td>
<td>Synthesis of Labelled cDNA</td>
<td>120</td>
</tr>
<tr>
<td>3.7.4iii</td>
<td>Purifying Labelled cDNA</td>
<td>122</td>
</tr>
<tr>
<td>3.7.5</td>
<td>Microarray Hybridisation Protocol</td>
<td>124</td>
</tr>
<tr>
<td>3.7.5i</td>
<td>General</td>
<td>124</td>
</tr>
<tr>
<td>3.7.5ii</td>
<td>Hybridisation</td>
<td>125</td>
</tr>
<tr>
<td>3.7.5iii</td>
<td>Washing the Microarray Slides</td>
<td>125</td>
</tr>
<tr>
<td>3.7.5iv</td>
<td>Generating images from the microarrays</td>
<td>126</td>
</tr>
<tr>
<td>3.7.5v</td>
<td>Data analysis</td>
<td>126</td>
</tr>
<tr>
<td>3.8</td>
<td>DRUG LEVEL ESTIMATION</td>
<td>127</td>
</tr>
<tr>
<td>3.8.1</td>
<td>General</td>
<td>127</td>
</tr>
<tr>
<td>3.8.2</td>
<td>Quercetin</td>
<td>127</td>
</tr>
<tr>
<td>3.8.2i</td>
<td>Extraction</td>
<td>127</td>
</tr>
<tr>
<td>3.8.2ii</td>
<td>Enzyme Hydrolysis Protocol</td>
<td>128</td>
</tr>
<tr>
<td>3.8.2iii</td>
<td>Whole Metabolites Protocol</td>
<td>128</td>
</tr>
<tr>
<td>3.8.2iv</td>
<td>High Performance Liquid Chromatography (HPLC)</td>
<td>128</td>
</tr>
<tr>
<td>3.8.3</td>
<td>Aspirin</td>
<td>128</td>
</tr>
<tr>
<td>3.9</td>
<td>STATISTICAL ANALYSIS</td>
<td>130</td>
</tr>
<tr>
<td>4.</td>
<td>RESULTS</td>
<td>131</td>
</tr>
<tr>
<td>4.1</td>
<td>VALIDATION STUDY</td>
<td>131</td>
</tr>
<tr>
<td>4.1.1</td>
<td>General</td>
<td>131</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Histology</td>
<td>133</td>
</tr>
</tbody>
</table>
4.1.3 CpG Island Methylation 139
4.1.3i ESR-1 139
4.1.3ii p16 139
4.1.3iii HPP1 141
4.1.4 Global Gene Transcription 144

4.2 INTERVENTION STUDY 148
4.2.1 General 148
4.2.1i Pre-Initiation Study 148
4.2.1ii Long-term Study 148
4.2.2 Serum Drug Concentrations 151
4.2.3 Oesophageal Cancer and Barrett’s Oesophagus 152
4.2.3i General 152
4.2.3ii Pre-Initiation vs Post-Initiation Intervention 152
4.2.3iii Cancer and Barrett’s Incidence 154
4.2.4 CpG Island Methylation 156
4.2.4i General 156
4.2.4ii ESR-1 156
4.2.4iii p16 158
4.2.4iv HPP1 158

5. DISCUSSION 161
5.1 VALIDATION STUDY 161
5.1.1 Morphological Changes in the Rat Model 161
5.1.2 CpG Island Hypermethylation 163
5.1.3 Gene Transcription 168
5.1.4 Summary 170

5.2 INTERVENTION STUDY 171
5.2.1 The Effect of Aspirin and Quercetin 171
5.2.2 Drug Dosing 174
5.2.3 CpG Island Hypermethylation and Treatment 176
5.2.4 Summary 179
APPENDIX

7.1 Rat Diets

7.1.1 Semi-Synthetic and Experimental Rat Diets

7.1.2 Components of the Mineral Mix

7.1.3 Components of the Vitamin Mix
FIGURES AND TABLES

Figure 1. Incidence of oesophageal cancer in the West (1975-97) 15
Figure 2. The metaplasia–dysplasia–adenocarcinoma sequence 18
Figure 3. COX and the arachadonic acid metabolic pathway 58
Figure 4. The chemical structure of quercetin 66
Figure 5. Graphical illustration of the anticarcinogenic effect of quercetin in vivo 69
Figure 6. Surgical models of OAC in the rat 73
Figure 7. Flow diagram of Validation Study design 86
Figure 8. Flow diagram of Intervention Study design 91
Figure 9. Photographs of oesophagojejunostomy in the rat 101
Figure 10. Photographs demonstrating ‘Swiss roll’ method of harvesting rat oesophagus for histological analysis 104
Figure 11. Virtual gel and graphical illustration of ‘good quality’ total RNA obtained using Agilent’s 2100 bioanalyser 121
Figure 12. Overview of the cDNA labelling procedure 123
Figure 13. Cause of death in rats that died unexpectedly in the Validation Study 132
Figure 14. Weight gain of operated rats in the Validation Study 132
Figure 15a. Graphical illustration of the morphological changes in the rat oesophagus following oesophagojejunostomy in the Validation Study 135
Figure 15b. Graphical illustration of the morphological changes in the rat oesophagus following oesophagojejunostomy in the Validation Study 136
Figure 16. Photographs of the macroscopic changes in the rat oesophagus following oesophagojejunostomy in the Validation Study 137
Figure 17. Photographs of the microscopic changes in the rat oesophagus following oesophagojejunostomy in the Validation Study 138
Figure 18. CpG island hypermethylation of the ESR1 promoter at different time points following oesophagojejunostomy 140
in the Validation Study

Figure 19. CpG island hypermethylation of the p16 promoter at different time points following oesophagojejunostomy in the Validation Study

Figure 20. CpG island hypermethylation of the HPP1 promoter at different time points following oesophagojejunostomy in the Validation Study

Figure 21. Gene expression in BO in the rat compared to humans

Figure 22. Gene expression in OAC in the rat compared to humans

Figure 23. Weight gain of rats in different experimental groups in the Intervention Study

Figure 24. Graphical illustration of the effect of aspirin and quercetin on the incidence of BO and OAC in rats treated prior to onset of reflux compared rats commenced on treatment after reflux was established

Figure 25. Graphical illustration of the incidence of BO and OAC within the aspirin and quercetin treatment groups

Figure 26. The effect of aspirin and quercetin on CpG island hypermethylation of the ESR1 promoter in BO and OAC

Figure 27. The effect of aspirin and quercetin on CpG island hypermethylation of the p16 promoter in BO and OAC

Figure 28. The effect of aspirin and quercetin on CpG island hypermethylation of the HPP1 promoter in BO and OAC

Table 1. Genetic abnormalities reported in BO and OAC

Table 2. CpG island hypermethylation events reported to occur in BO and OAC

Table 3. Summary of studies included in a meta-analysis comparing medical treatment and anti-reflux surgery on incidence of OAC in patients with BO (Corey et al. 2003)

Table 4a. Summary of studies included in a meta-analysis investigating the potential protective effect of NSAIDs and aspirin against OC (Corley et al. 2003)

Table 4b. Odds Risk of developing OC with NSAID/aspirin use