

## 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 T/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

3.7.2	RNA Extraction	115
3.7.2i	General	115
3.7.2ii	Release of RNA from Tissues	116
3.7.2iii	Binding of RNA to Column	117
3.7.2iv	Washing the Column	117
3.7.2v	RNA Elution	117
3.7.3	Total RNA Quantification and Quality Control	118
3.7.3i	Preparation of Gel-Dye Mix	118
3.7.3ii	Preparation of Nano labchip	118
3.7.3iii	Running the Chip	119
3.7.4	RNA Preparation and Labelling	120
3.7.4i	General	120
3.7.4ii	Synthesis of Labelled cDNA	120
3.7.4iii	Purifying Labelled cDNA	122
3.7.5	Microarray Hybridisation Protocol	124
3.7.5i	General	124
3.7.5ii	Hybridisation	125
3.7.5iii	Washing the Microarray Slides	125
3.7.5iv	Generating images from the microarrays	126
3.7.5v	Data analysis	126
3.8	DRUG LEVEL ESTIMATION	127
3.8.1	General	127
3.8.2	Quercetin	127
3.8.2i	Extraction	127
3.8.2ii	Enzyme Hydrolysis Protocol	128
3.8.2iii	Whole Metabolites Protocol	128
3.8.2iv	High Performance Liquid Chromatography (HPLC)	128
3.8.3	Aspirin	128
3.9	STATISTICAL ANALYSIS	130
4.	RESULTS	131
4.1	VALIDATION STUDY	131
4.1.1	General	131
4.1.2	Histology	133

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 <del>69</del>
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

6	REFERENCES	180
7	APPENDIX	20 <del>92</del>
7.1	Rat Diets	20 <del>92</del>
7.1.1	Semi-Synthetic and Experimental Rat Diets	20 <del>92</del>
7.1.2	Components of the Mineral Mix	21 <del>003</del>
7.1.3	Components of the Vitamin Mix	21 <del>104</del>

## 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	142
Figure 20.	CpG island hypermethylation of the HPP1 promoter at different time points following oesophagojejunostomy in the Validation Study	143
Figure 21.	Gene expression in BO in the rat compared to humans	145
Figure 22.	Gene expression in OAC in the rat compared to humans	147
Figure 23.	Weight gain of rats in different experimental groups in the Intervention Study	150
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	153
Figure 25.	Graphical illustration of the incidence of BO and OAC within the aspirin and quercetin treatment groups	155
Figure 26.	The effect of aspirin and quercetin on CpG island hypermethylation of the ESR1 promoter in BO and OAC	157
Figure 27.	The effect of aspirin and quercetin on CpG island hypermethylation of the p16 promoter in BO and OAC	159
Figure 28.	The effect of aspirin and quercetin on CpG island hypermethylation of the HPP1 promoter in BO and OAC	160
Table 1.	Genetic abnormalities reported in BO and OAC	23
Table 2.	CpG island hypermethylation events reported to occur in BO and OAC	27
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)	42
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)	55
Table 4b.	Odds Risk of developing OC with NSAID/aspirin use	55