3. MATERIALS & METHODS

3.1 STUDY DESIGN - Validation study

3.1.1 GENERAL

A time course experiment was performed to assess the morphological and molecular changes that occur in the rat oesophagus in response to surgically induced enterooesophageal reflux. Following the 2-week acclimatisation period, rats were randomised into 7 groups of 10 animals after surgical induction of reflux disease. Each group was then randomised to a different time point for sacrifice. The first group of animals was sacrificed at 4 weeks post-operatively. Subsequent groups were sacrificed at 8, 12, 16, 20, 24, and 28 weeks post-operatively. The entire oesophagus together with a short segment of afferent and efferent jejunal loop was harvested from the sacrificed rats and used for morphological and molecular analysis.

An additional 16 animals that had not undergone surgery were followed for up to 28 weeks after the initial 2-week acclimatisation period to obtain normal age matched oesophageal tissue for analysis. Three of these animals were sacrificed at 4 weeks, and 3 at 16 weeks to provide aged matched tissue for the molecular analysis at these time points. The remaining rats were sacrificed at 28 weeks.

During the study period animals were housed individually and fed a powdered semisynthetic diet and water *ad libitum*. Any animal that appeared ill was observed closely for 48 hours. If no improvement in its general condition was observed during this time it was killed and autopsied. Rats were weighed monthly, and all animals received monthly intramuscular iron dextran

(50mg/kg) to prevent anaemia due to reduced iron absorption. A flow diagram of the validation study design is shown in Figure 7.

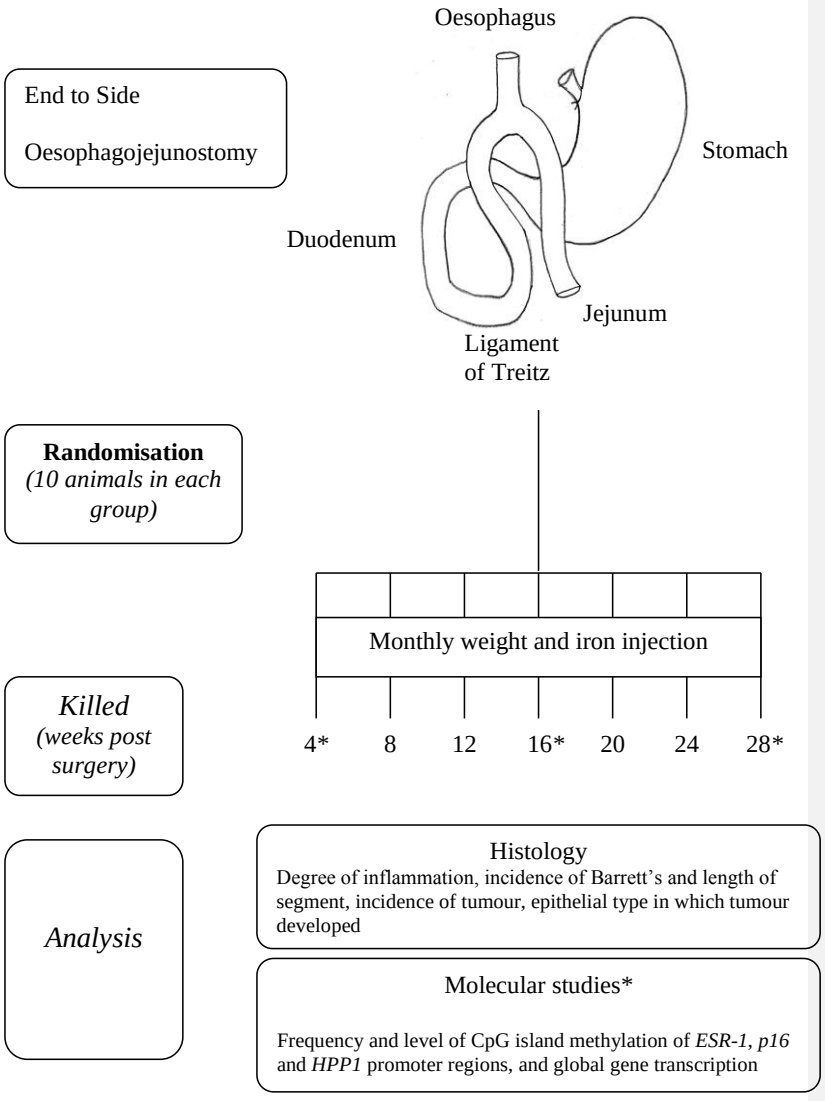

Figure 7. Validation study design. A flow chart demonstrating randomisation, time course, and study end points

3.1.2 HISTOLOGICAL ANALYSIS

The histological changes in the oesophagus of all rats in the study were determined after sacrifice by light microscopy once the tissue specimen had been sectioned and stained with haemotoxylin and eosin.

3.1.3 MOLECULAR ANALYSIS

3.1.3i Methylation Studies

Genomic DNA from the proximal oesophagus (normal squamous epithelium), distal oesophagus (Barrett's oesophagus), and tumour (if present) was isolated from frozen tissue specimens of all animals killed at 4, 16 and 28 weeks after surgery. The DNA from the proximal oesophagus of each rat served as an internal control.

The CpG island methylation status of the *ESR-1* promoter region was determined using a novel quantitative method based on the polymerase chain reaction centered on the locus of interest within the *ESR-1* promoter. Subsequent determination of the CpG island methylation status of the *p16* and *HPP1* promoter regions was performed on the genomic DNA isolated from this study by Dr N. Belshaw, a post doctorate research scientist working in the Department of Gastrointestinal Research at The Institute of Food Research, Norwich Research Park. These results are included in this thesis as a point of interest and for completeness.

3.1.3ii. Global Gene Transcription

Three animals were randomly selected from each group of operated animals killed at 4 weeks, 16 weeks and 28 weeks for analysis of global gene transcription. Three non-operated animals were randomly selected and killed at each of these time points to provide age matched controls. Total RNA from the distal oesophagus (Barrett's oesophagus), and tumour (if present) was isolated.

Microarray studies were performed using Agilent's rat cDNA arrays that contain 14500 gene transcripts (Agilent Technologies Ltd). RNA extracted from the distal oesophagus (Barrett's oesophagus) and tumour (if present) from each experimental animal was analysed individually against RNA extracted from distal oesophagus of the age matched non-operated controls. At each time point a direct comparison of global gene transcription in the distal oesophagus (Barrett's oesophagus) and tumour (if present) in rats that had undergone surgery was made with the controls. Analysis of gene transcription at different time points after surgery, and in both Barrett's tissue and adenocarcinoma allowed the pattern of transcriptional dysregulation that occurred with disease progression to be observed.

A thorough literature review was conducted to identify all genes implicated in the pathogenesis of Barrett's oesophagus and oesophageal adenocarcinoma in humans. Those genes reported to undergo transcriptional dysregulation in humans that were represented on the rat microarray were identified and their level of expression determined at each time point.

3.2 STUDY DESIGN - Intervention Study

3.2.1 GENERAL

Following the observation that all rats have severe inflammatory changes of the distal oesophagus and histological evidence of Barrett's oesophagus 4 weeks after surgical induction of reflux disease (See Validation Results page 133-138), the intervention study was designed to assess the potential chemopreventive effect of the experimental drugs on both disease initiation and progression.

Six-week old male Sprague Dawley rats were randomised into two experimental groups, a *pre-initiation* and a *long-term* group. Rats in the *pre-initiation* group were fed powdered semisynthetic rat chow containing aspirin or quercetin during the 2-week acclimatisation period prior to surgical induction of reflux disease. This diet was recommenced immediately after surgery. Rats in the *long-term* group were fed the semisynthetic diet without the addition of the experimental chemopreventive agents both during the 2-week acclimatisation period and until 4 weeks after surgery when reflux had been firmly established. After this time aspirin, quercetin or placebo was added to the rat chow.

3.2.1i. Pre-Initiation Group

Thirty-two 6-week old male Sprague-Dawley rats were randomised into 2 groups of 16 animals. Each group of rats was then assigned to receive either aspirin or quercetin containing experimental diets. After 2 weeks all rats underwent oesophagojejunostomy. Following recovery from surgery all rats were recommenced on the experimental diet to which they were assigned and this was continued for the duration of the experiment. All rats were sacrificed at

28 weeks +/- 1 week after surgery. At sacrifice, the entire oesophagus together with a short segment of afferent and efferent jejunal loop was harvested from the sacrificed rats and used for morphological and molecular analysis.

3.2.1ii. Long-Term Group

Four weeks after oesophagojejunostomy 105 rats were randomised into 3 groups of 35 animals. Each group was then assigned to receive 1 of the 3 experimental diets containing aspirin, quercetin or placebo. The rats continued on the diet to which they had been assigned for the remainder of the experiment. All rats were sacrificed at 28 weeks +/- 1 week after surgery. At sacrifice the entire oesophagus together with a short segment of afferent and efferent jejunal loop was harvested from the sacrificed rats and used for morphological and molecular analysis. An additional 16 rats that had not undergone surgery were sacrificed 28 weeks after the initial 2-week acclimatisation period to obtain normal age matched oesophageal tissue for analysis.

During the study period all animals in both the *pre-initiation* and *long term* groups received monthly intramuscular iron dextran (50mg/kg) (Leodex 20%; Leo laboratories Ltd) to prevent anaemia due to reduced iron absorption. In addition, all rats were weighed monthly. Any animal that appeared ill was observed closely for 48 hours. If no improvement in its general condition was observed during this time it was killed and autopsied. A 5ml-blood sample was obtained from all rats at sacrifice for estimation of serum drug levels.

A flow diagram for the intervention study design is shown in Figure 8.

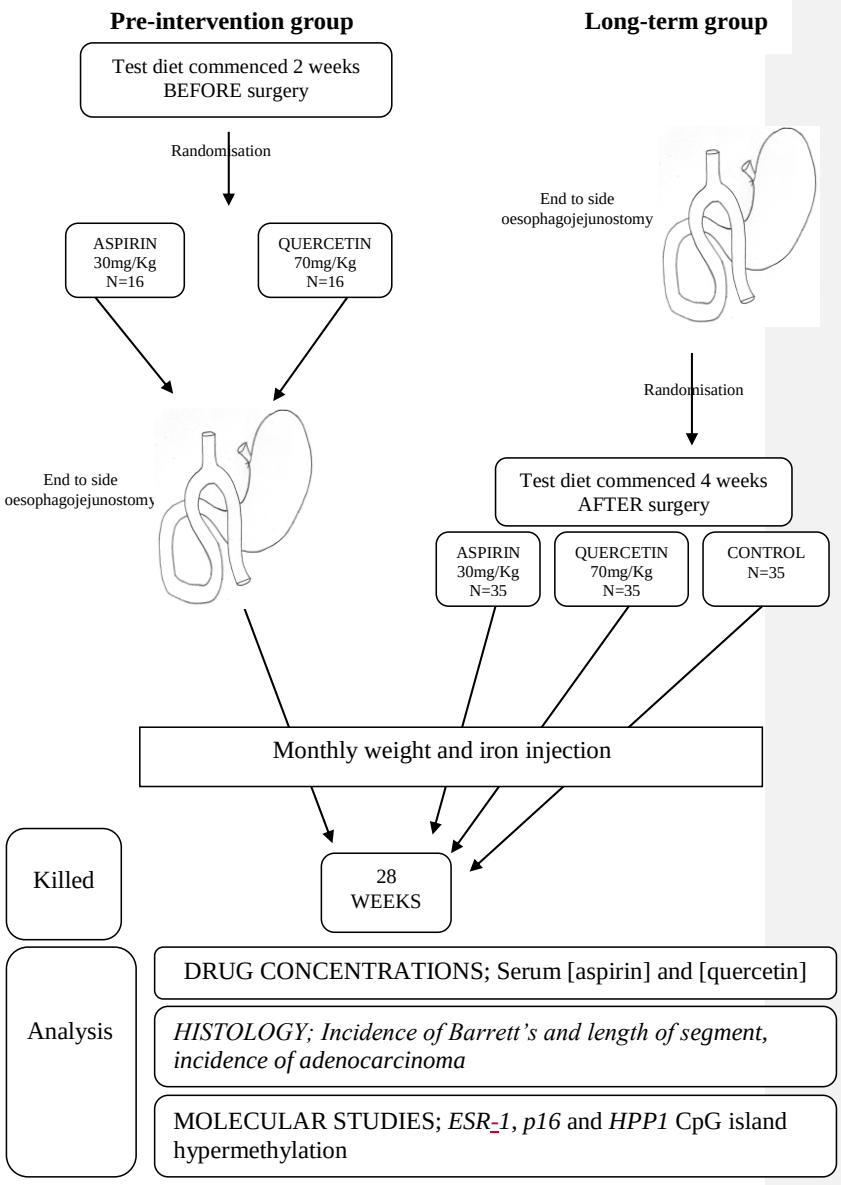

Figure 8. Intervention study design. A flow chart demonstrating randomisation and study end points

3.2.2 SAMPLE SIZE AND POWER CALCULATION

The sample sizes for the long term study were calculated using the on-line sample size calculator at www.dssresearch.com.

3.2.2i Aspirin

Based on the findings of Buttar et al, 2002 who reported that 25% of rats treated with the COX-2 inhibitor MF-tricyclic developed oesophageal adenocarcinoma compared to 55% of controls, the following variables were used in the calculation:

Sample 1	55%	% OAC expected in sample 1 (Control)
Sample 2	25%	%OAC expected in sample 2 (Aspirin)
Alpha error level	5%	Probability of Type 1 error (represents 95% CI)
Beta error level	20%	Probability of Type 2 error (Power of 0.8)

On the basis of these parameters the sample size required was 32 for both the aspirin and control groups.

3.2.2ii Quercetin

There are no previous studies reporting the reduction in OAC incidence attributable to quercetin in either human or animal studies on which to base a sample size calculation. However, Cheong et al demonstrated that quercetin is a potent inhibitor of COX-2 in vivo in the OAC cell line OE33 (Cheong et al

2004). The inhibitory effect of quercetin on cell growth and proliferation was significantly greater than the selective COX-2 inhibitors nimesulide and NS-398.

Based on these findings, a sample size of 32 was also used in the quercetin group.

3.2.3 DRUG DOSING - Dose selection

3.2.3i Aspirin

The dose of aspirin was selected based on a previous report that quantified the anti-inflammatory activity of aspirin in an in-vivo model of acute inflammation in male Sprague-Dawley rats (Vinegar et al 1976). The median value of the individual effective dose (ED 50%) was 31.0mg/Kg +/-7.0mg/Kg body weight in this model. An aspirin dose of 30mg/Kg body weight was therefore selected for this study.

3.2.3ii Quercetin

The dose of quercetin was selected based on previous studies in our laboratory that quantified the effect of different levels of dietary quercetin on the suppression of intestinal crypt cell proliferation in the small and large bowel of male Wistar rats (Gee et al 2002). A dietary quercetin level of 1g/Kg of diet (equivalent to 70mg/Kg body weight) was associated with the greatest decrease in crypt cell mitoses, and was therefore selected for this study.

3.2.3iii Drug Administration

Drug dosing was achieved by feeding 20g aliquots of diet (the average daily amount of food consumed by an adult Sprague Dawley rat) every 24h mixed with the test drug. Based on a body weight of 330g (the mean weight achieved by male Sprague Dawley rats over a 28 week period after oesophagojejunostomy) each 20g aliquot of food was mixed with 10mg of aspirin or 25mg quercetin to achieve a daily dose of approximately 30mg/Kg and 70mg/Kg respectively.

3.2.3iv Drug Blood Level Estimation

Blood samples were taken from all rats by cardiac puncture immediately after they were killed to confirm the test drugs had been absorbed and determine their plasma or serum concentration.

3.2.4. HISTOLOGICAL ANALYSIS

The histological changes in the oesophagus of all rats in the study were determined after sacrifice by light microscopy once the tissue specimen had been sectioned and stained with haemotoxylin and eosin.

3.2.5. METHYLATION STUDIES

Genomic DNA from jejunum, proximal oesophagus (normal squamous epithelium), distal oesophagus (Barrett's oesophagus), and tumour was isolated from frozen tissue specimens collected from randomly selected animals in each experimental group within the *long-term* study to determine the degree of CpG island methylation of the *ESR-1* promoter region. The DNA from the jejunum

and proximal oesophagus of each rat served as an internal control. .Again,Subsequent determination of the CpG island methylation status of the *p16* and *HPP1* promoter regions was performed on the genomic DNA isolated from this study by Dr N. Belshaw, a post doctorate research scientist working in the Department of Gastrointestinal Research at The Institute of Food Research, Norwich Research Park. Again, and these results are included in this thesis as a point of interest and for completeness.

The CpG island methylation levels of the ESR1, p16 and HPP1 gene promoter regions were determined using the same novel quantitative method employed in the validation study.

3.3 RAT MODEL

3.3.1 GENERAL

Six-week old male Sprague-Dawley rats were purchased from Tucks and sons plc. The animals were housed 2 per cage and kept under standard laboratory conditions which comprised a 12h light/dark cycle with the room temperature at 22 +/- 2ºC, room humidity at 55% +/- 5%, and 15 to 20 air changes/hour. After a 2-week acclimatisation period, during which time all animals received a powdered semisynthetic chow and had access to water *ad libitum*, the rats underwent oesophagojejunostomy using Levrat's technique to induce enterooesophageal reflux (Levrat et al 1962). The rats were not starved prior to surgery, having free access to both chow and water until induction of anaesthesia. All rats were weighed immediately before surgery.

3.3.2 SURGICAL INDUCTION OF REFLUX DISEASE

3.3.2i Pre-Operative Handling and Stress Reduction

Following a number of peri-operative deaths early in the study it became clear that rats that were stressed prior to being operated on were more likely to experience complications during surgery than those that were not. To reduce the rats stress levels to a minimum pre-operatively, ambient noise in the holding room and operating theatre was kept to an absolute minimum during operating days. Animals were retrieved from the holding room quietly with the minimum amount of handling and slow movements. The carry container used to transport the rats from the holding area to the operating area was cleaned after each journey. If a rat was removed from a cage housing 2 animals, the paired animal was not operated on the same day. Finally, if any difficulty was experienced in

inducing anaesthesia the rat was returned to the holding area and the operation postponed to another day.

3.3.2ii Anaesthetic

General anaesthesia was induced using isoflourane (99.9%, 0.5mL/L volume; Schering-Plough Animal Health) by placing the rat in an airtight container containing a paper towel soaked with 1ml of the volatile anaesthetic agent for approximately 45 seconds. Successful induction of anaesthesia was indicated by cessation of movement, cessation of blinking and slow regular respiration. Generally, but not invariably there was flexion at the hip and knee. Maintenance anaesthesia was initially provided by an intramuscular injection of 0.5ml 2% xylazine hydrochloride (Millpledge Pharmaceuticals) and 2.5ml ketamine (Fort Dodge Animal Health Ltd) into the gluteal region both administered once the rat was asleep. Subsequently maintenance of anaesthesia was provided by intermittent intra-peritoneal boluses of 0.5ml ketamine during the operation, based on the respiratory rate of the rat.

All animals received a 1ml subcutaneous bolus of warmed Hartmann's solution (Baxter Healthcare Ltd), and 50mg of iron dextran (Leodex 20%; Leo laboratories Ltd) intramuscularly at induction to compensate for water/blood loss during the surgical procedure. 5mg/kg enrofloxacin (Baytril; Bayer Healthcare) was given prophylactically to prevent post-operative intra-abdominal sepsis.

3.3.2iii. Oesophagojejunostomy

Following induction of anaesthesia, the abdomen was shaved from the external genitalia to the mid thorax using electrical shears. The rat was placed supine on a polystyrene board, and held in place with elastic bands around both fore and hind limbs. The operative field was sterilised using an alcohol wipe, and draped to create a sterile field.

The operation was performed through an upper midline incision made with scissors and extending from the xiphisternum distally for approximately 3cm, taking care not to puncture the diaphragm. The left lobe of the liver was retracted laterally to the right to expose the stomach and distal oesophagus using a cotton bud soaked in Hartmann's solution. Any congenital peritoneal adhesions between the liver and stomach were divided to facilitate access.

After identifying the gastroesophageal junction, a plane was developed using forceps between the left distal oesophagus and surrounding fatty tissue containing the left vagus nerve. This plane was extended behind the oesophagus and a window created between the right distal oesophagus and the fatty tissue containing the right vagus nerve. This window was extended proximally using a vein clamp, and the distal oesophagus clamped approximately 0.5cm from the gastroesophageal junction (GOJ). The oesophagus was ligated at the GOJ using a 2/0 silk ligature, and transected just proximal to the ligature, with preservation of the vagal trunks. Any detritus at the proximal and distal transected margins of the oesophagus was removed with forceps. After pushing the stomach back into the left upper quadrant under the

proximal oesophageal stump, 4 sutures were placed at the left, right, anterior and posterior aspect of the proximal oesophageal stump using 7/0 prolene. The clamp was removed, and a further 4 sutures were placed in the proximal oesophageal stump equidistant between the corner sutures using 7/0 prolene.

The jejunum was identified and followed back to the Ligament of Treitz. The most proximal jejunal loop was pulled up to the proximal oesophageal stump, and an enterotomy site selected that would provide the shortest length of jejunum between the Ligament of Treitz and oesophagus whilst allowing a tension free anastomosis. This distance was approximately 1cm distal to the Ligament of Treitz. A vein clamp was applied to the jejunum proximal to the enterotomy site, and a 0.8-10mm enterotomy fashioned in the antimesenteric border of the jejunum. Any detritus within the small bowel at the enterotomy site was removed using a cotton bud soaked in Hartmann's solution. The right and left oesophageal corner sutures were placed at the proximal and distal corners of the enterotomy respectively. The posterior oesophageal suture was placed at the mid point in the posterior wall of the enterotomy, with the remaining posterior wall sutures placed equidistant between the corner and posterior enterotomy sutures. After tying the corner sutures, the posterior wall sutures were tied forming the posterior wall of the anastomosis. The anterior oesophageal suture was then placed at the mid point of the anterior enterotomy wall, with the remaining anterior wall sutures placed equidistant between the corner and anterior enterotomy sutures. The anterior sutures were tied forming the anterior wall of the anastomosis. The vein clamp was removed. Occasionally additional sutures were required to secure the anastomosis and

these were placed wherever they were needed. The anastomosis was placed under the left lobe of the liver, and the abdominal wall closed by mass closure using a 3/0 silk suture. The skin was closed with interrupted stitches using a 3/0 vicryl suture (Ethicon, Johnson and Johnson medical Ltd). Photographs of the operation are shown in Figure 9.

To prevent excessive drying of the abdominal viscera intra-operatively, the abdominal cavity and contents were regularly brushed with Hartmann's solution using a cotton bud during the procedure.

3.3.3 RECOVERY

Following surgery, rats were housed in a quiet darkened room separate to the rest of the animal house. This room was maintained at the higher temperature of 25ºC +/-2 compared to standard conditions, but at the same humidity and air change level.

The rats were placed individually in wire bottom cages in the left lateral position under a heating lamp until they recovered from the anaesthetic. To minimise the risk of aspiration after surgery as little pressure as possible was applied around the abdomen when handling the rats. In addition, rats were transported from the operating theatre to the recovery room vertically in the head up position, and approximately 20 degrees of head up was maintained during recovery by propping one end of the wire bottom cage up on blocks. Once ambulant, the rats were maintained on a dilute Ensure plus solution (Abbott Nutrition, Kent, England) containing 1mg/ml paracetamol (Paracetamol

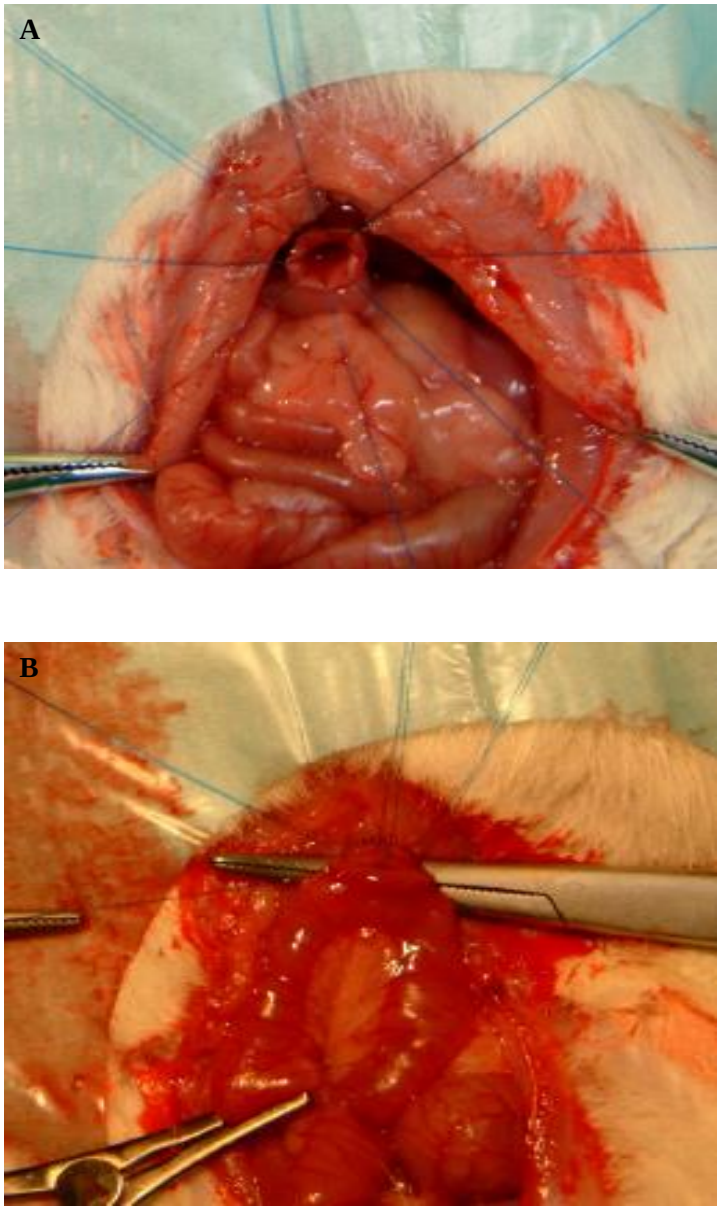

Figure 9. Oesophagojejunostomy in the rat. (**A**) Typically eight sutures are used to fashion the oesophagojejunal anastomosis. The sutures are placed in the distal oesophagus following ligation and transection of the oesophagus immediately above the gastroesophageal junction. (**B**) Placement of the anterior sutures in the oesophagojejunal anastomosis after completion of the posterior wall.

Elixir Paediatric BP; Thornton and Ross, Huddersfield, England). On the third post-operative day feeding was recommenced with the powdered semisynthetic chow, and the rats were moved back into the main housing area and kept under standard laboratory conditions for the duration of the experiment.

3.4 TISSUE HARVEST

Upon reaching the designated time point for sacrifice, or following 48 hours of illness with no clinical improvement, animals were killed by placing them in a sealed container and administering an overdose of isoflourane (99.9%, 0.5mL/L volume; Schering-Plough Animal Health) according to Schedule 1 to the Animals (Scientific Procedures) Act 1986.

After sacrifice the rat was laid out supine and pinned to a polystyrene board. The chest and abdomen were opened with scissors in the midline by making an incision from the suprasternal notch to the pubic bone. The incision was then extended proximally to the larynx. The entire oesophagus was resected from the level of the larynx together with 1cm of the afferent and efferent jejunal loop forming the anastomosis.

The oesophagus was opened longitudinally along the anterior surface and the macroscopic changes documented including the presence and proximal extent of inflammation. If tumour was present its site was noted, and the dimensions of the tumour measured in three planes. The oesophagus was then bisected longitudinally with the line of incision dividing any visible tumour. One half of the oesophagus was further divided longitudinally, and the two portions rolled in the manner of a 'Swiss roll' as demonstrated in Figure 10. The rolled tissue was embedded in paraffin blocks after being fixed in 10% formalin for 24hours. Preparing the tissue specimens in this way facilitated histological analysis of the entire length of oesophageal epithelium in continuity.

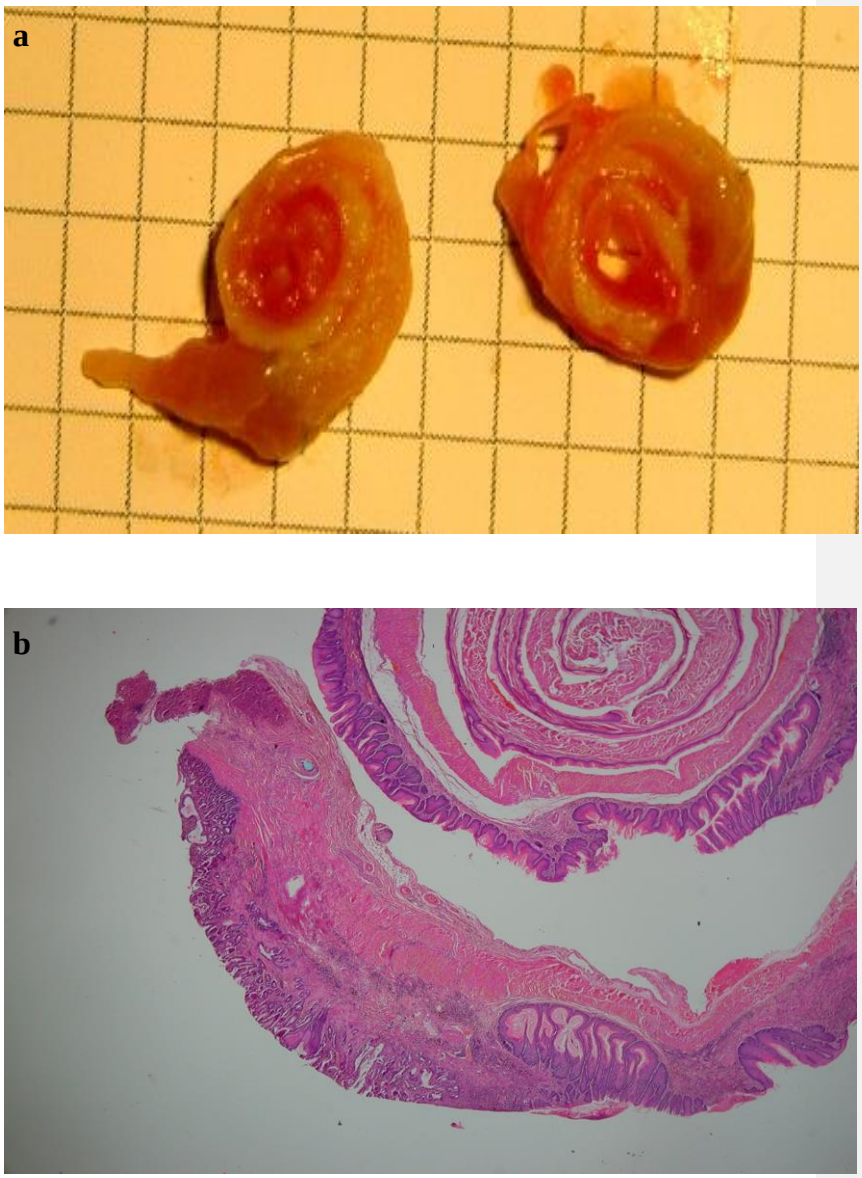

Figure 10. Rolling the bisected rat oesophagus in the manner of a 'Swiss roll' prior to fixation (**a**) allowed the entire length of the oesophagus to be examined in continuity during microscopy (**b**).

The remaining oesophagus was used for molecular analysis. The distal oesophagus was cut 2mm proximal to the anastomosis. Any visible tumour was dissected off the specimen and triplicate tissue samples were taken from tumour, the distal 10mm of oesophagus, and the proximal oesophagus. From each set of triplicate biopsy specimens one sample was fixed in 10% formalin for 24 hours and then embedded in paraffin to provide histological verification of the tissue pathology used in the molecular analysis. Of the two remaining samples from each region, one was snap frozen and stored at –80ºC for DNA extraction and CpG island methylation analysis. The final tissue specimen was snap frozen in RNA later (Applied Biosystems/Ambion, Inc) and stored at –80ºC for RNA extraction and estimation of global gene transcription by microarray analysis.

3.5 HISTOLOGICAL ANALYSIS

The paraffin blocked rolled specimens and the tissue biopsies taken for pathological verification in the molecular studies, were cut into 6μm sections using a microtome and stained with haematoxylin and eosin. All tissue specimens were examined independently by two Consultant histopathologists at the Norfolk and Norwich University Hospital with a special interest in Barrett's oesophagus and oesophageal adenocarcinoma who were blinded to the group of origin of each tissue sample. Where there was disagreement about a tissue diagnosis, the tissue sample was re-examined by both histopathologists together and a consensus diagnosis was reached.

A diagnosis of Barrett's oesophagus was made if columnar epithelium with intestinal metaplasia (columnar cells with a brush border and the presence of goblet cells) was present proximal to the anastomosis. Carcinoma was diagnosed if dysplastic cells invaded through the basement membrane, and classified as either adenocarcinoma or squamous carcinoma. The predominant epithelial type in which carcinoma had developed was also recorded.

3.6 CpG ISLAND METHYLATION ANALYSIS

3.6.1 DNA EXTRACTION

DNA extraction was performed using Sigma's GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA).

To reduce the risk of DNA contamination during the procedure, all disposable equipment including scalpels, eppendorfphs, pestlecels, and pipettes were sterilised prior to the experiment. This was achieved by autoclaving under standard conditions of 15 pounds/sq inch (1 atmosphere) above atmospheric pressure (760 mm Hg), at 121°C for 15 minutes. The components of the Genelute kit were sterilised by the manufacturer.

To minimise DNA degradation during the extraction procedure, the samples were kept cool by placing them on ice wherever the method allowed and repeated freeze-thaw episodes were avoided.

3.6.1i Release of DNA from Tissues

The frozen tissue samples were transferred to an icebox and allowed to thaw. After weighing, approximately 20mg of tissue from each sample was transferred to a sterile 2ml eppendorfph and mashed for 5 minutes using a small sterile pestle. Once mashed, 180ul lysis buffer (kit number B6678) and 20ul Proteinase Kk was added to each sample. The samples were then incubated overnight at 55^{o}C in an electric oven.

After removal from the oven, 200µl of lysis solution (kit number B8803) was added each sample. The samples were mixed thoroughly for 15 seconds using a vortex, and immediately placed in a heating block and incubated at 70ºC for 10 minutes, following which they were placed back on ice to cool.

3.6.1ii Binding of DNA to Column

Once cool, 200µl absolute ethanol (96-100%) was added to each sample and mixed thoroughly for 10 seconds using a vortex. The entire sample was then transferred to a binding column (Genelute Nucleic Acid Binding Column) using a sterile wide bore pipette to avoid shearing the DNA during transfer, and spun in a centrifuge at 6500g for 1 minute.

3.6.1iii Washing the Column.

After discarding the binding column collection tube and replacing it with a new one, 500µl wash solution (kit) was added to the column using a sterile pipette. The column was then re-spun in the centrifuge at 6500g for 1 minute. To ensure thorough washing of the column, this procedure was repeated 2 more times. On the final wash, the column was spun in the centrifuge at 12000g for 3 minutes to dry the column.

3.6.1iv DNA Elution

A new collection tube was placed on the binding column, and after adding 200µl elution solution (kit) to the column, the column was spun in the centrifuge at 6500g for 1 minute. To maximise the amount of DNA obtained

from the column, this procedure was repeated but without changing the collecting tube.

This resulted in approximately 400µl of elution solution containing purified DNA in the final sample.

The extracted DNA was stored at –20ºC.

3.6.2 QUANTIFICATION AND QUALITY CONTROL OF EXTRACTED DNA

The concentration of DNA extracted from each sample was determined by spectrophotometry. 50µl of the extracted DNA sample was transferred to a clean quartz microcurvette. The spectrophotometer was blanked~~zeroed~~ using plain elution solution. The ultra-violet spectrum of the DNA sample was then measured, and the absorbence of the DNA solution at 260nm and 280nm determined. If the absorbence at these wavelengths was above the linear range of the spectrophotometer (0.1-1.0), the DNA sample was serially diluted with elution solution until its absorbence fell within these limits. An absorbence of 1.0 at a wavelength of 260nm corresponded to a DNA concentration of 50µg/ml. Therefore, the concentration of the DNA present in the sample was calculated using the following equation:

[DNA] µg/ml = absorbence at 260nm x 50 (x dilution factor if serially diluted)

The purity of DNA was determined by calculating the ratio of absorbence at 260nm to the absorbence at 280nm. A ratio of 1.5-2 represented pure DNA.

DNA samples that were not pure were rebound to a fresh DNA binding column and washed as described in 3.6.1

3.6.3 BISULPHITE MODIFICATION

Bisulphite specifically deaminates unmethylated cytosine bases to uracil, whilst methylated cytosines remain unconverted.

The extracted genomic DNA was bisulphite modified using the method described by Raizis (Raizis et al 1995). 2 µgs of DNA was desulphonated by mixing with 3µl of 2M NaOH, made up to a total volume of 20µl, and incubated at 37°C for 15 minutes. The bisulphite solution was prepared by dissolving 3.8g of sodium bisulphite in 6mls sterile water, adding 1.4mls of 0.75M Hydroquinone solution and adjusting the pH with 280µls of 10M NaOH. 400µls of the bisulphite solution was added to the denatured DNA and incubated at 50°C for 4 hours.

3.6.4 PURIFICATION OF BISULPHITE MODIFIED DNA

DNA was recovered using the QIAquick kit (Qiagen, Crawley, UK) followed by elution in 40µls Elution buffer. 1.2µl 10M NaOH was added to the sample, and incubated at 37 °C for 15 minutes to desulphonate the DNA. The solution was neutralised by adding 28µls of 10M Ammonium acetate. 1µl glycogen was added to the sample to aid visualisation of the precipitated DNA. The DNA was then precipitated by adding 210µl cold absolute ethanol (96-100%) to the sample, and storing at –70°C overnight. The precipitated DNA was formed into a pellet by spinning the sample at –10°C in a centrifuge at 23000g for 30 minutes. After carefully removing the supernatant using a pipette, the DNA pellet was washed by adding 0.5ml 70% ethanol and spinning the sample at –

10ºC in a centrifuge at 23000g for 5 minutes. Again, the supernatant was removed

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carefully with a pipette, and the DNA pellet allowed to air dry. Once dry, the DNA was re-suspended in 10µl Qiaquick elution buffer.

The bisulphite modified DNA was stored at –70°C.

3.6.5 ESR-1

3.6.5i PCR Amplification

A PCR reaction was performed on the bisulphite-modified DNA in order to amplify the promoter region of the estrogen receptor α gene (*ESR-1*) using the following primers: GAATGGTTTTGTTGTATTAGATTTAAGGGAA (forward primer) and CTCCAAATAATAAAACACCTAATAACCATAC (reverse primer). Each PCR reaction contained 4pmol of each of the forward and reverse primers, 10µl of HotStarTaq master mix (Qiagen), supplemented with $MgCl_2$ to 0.5mM and Bovine serum albumin to 1mg/ml. The following cycling conditions were used: 1 cycle of 95°C for 15 minutes, 35 cycles of 95°C for 30 seconds, 61°C for 1 minute (annealing temperature), 72°C for 1 minute followed by a 5 minute extension step of 72°C. Products from the PCR reaction were then used for quantitative methylation specific PCR (MSP) reactions.

3.6.5ii Quantitative MSP Reaction

Bisulphite modification results in the creation of methylation-dependent sequence differences in DNA. These can be quantified by fluorescence-based real-time PCR with SYBR green as reporter. The following primers overlapping potential methylation sites (CpG dinucleotides) were used to quantify the number of methylated alleles present in the amplified pool of

products from the initial PCR with bisulphite-modified DNA: CGTCGTTTACGAGTTTAACGTC (forward primer) and GTAAACCTACGCGTTAACGACG (reverse primer). Cycling conditions for this reaction were as follows: 1 cycle of 50°C for 10 seconds, 1 cycle of 95 °C for 15 minutes, and 40 cycles of 95°C for 30 seconds, 68°C for 30 seconds (annealing temperature), 72°C for 30 seconds. The total number of alleles (methylated and unmethylated) present in the amplified pool were determined using a semi-nested PCR with GGTGTTAATAGTTTGGGGGTTT as forward primer and the reverse primer used in the initial amplification from bisulphite-modified DNA described above. Cycle conditions for this reaction were as follows: 1 cycle of 50°C for 10 seconds, 1 cycle of 95 °C for 15 minutes, and 40 cycles of 95°C for 30 seconds, 63°C for 30 seconds (annealing temperature), 72°C for 30 seconds. Initial template quantity was derived from the cycle number at which the fluorescence signal crosses a threshold in the exponential phase of the PCR reaction (Ct). Standards at serial dilutions were included on each plate. These were known quantities of plasmid (pCR4-TOPO, Invitrogen) containing an inserted PCR fragment amplified using the primers described above from bisulphite-modified, SssI-methylase treated rat DNA. All samples were measured in triplicate. Methylation indices were calculated by dividing the number of methylated fragments by the total number of fragments for each sample.

The same process was repeated for the *p16* and *HPP1* genes as outlined below.

3.6.6 p16

3.6.6i PCR Amplification

The PCR reaction performed on the bisulphite-modified DNA in order to amplify the promoter region of the *p16* gene used the following primers: GTTTTTTTAAAATTTGGGGTTTGTT (forward primer) and CCCCTTAAAATACTAAACTCCTTTC (reverse primer). Each PCR reaction contained the same reagents in the same quantity as the *ESR-1* reaction. The cycling conditions were also the same as *ESR-1* with the exception of the annealing temperature which was 59 ^{o}C.

3.6.6ii Quantitative MSP Reaction

The following primers overlapping potential methylation sites (CpG dinucleotides) were used to quantify the number of methylated alleles present in the amplified pool of products from the initial PCR with bisulphite-modified DNA: GAGGAGGAGAGATTTCGATTTC (forward primer) and AAAATACTAAACTCCTTTCGAACG (reverse primer). The cycling conditions were the same as *ESR-1* with the exception of the annealing temperature which was 63 ^{o}C. The total number of alleles (methylated and unmethylated) present in the amplified pool were determined using a semi-nested PCR with AAACACCATAAAAAAACACATCTAAA as forward primer and GTTGGGAGGAGGAGAGATTT as reverse primer. Again the cycling conditions were the same as *ESR-1* with the exception of the annealing temperature which was 59 ^{o}C.

3.6.7 HPP1

3.6.7i PCR Amplification

The PCR reaction performed on the bisulphite-modified DNA in order to amplify the promoter region of the *HPP1* gene used the following primers: CAAAATATCCAACTACTACACTACC (forward primer) and GGTTGAGTTTAGTTATTGGATGTTGTT (reverse primer). Each PCR reaction contained the same reagents in the same quantity as the *ESR-1* reaction. The cycling conditions were also the same as *ESR-1* with the exception of the annealing temperature which was 55 ^{o}C.

3.6.7ii Quantitative MSP Reaction

The following primers overlapping potential methylation sites (CpG dinucleotides) were used to quantify the number of methylated alleles present in the amplified pool of products from the initial PCR with bisulphite-modified DNA: CGGAGATTATGCGTTTTTTTGGTC (forward primer) and CAACGACTTCTAAAAAACACGAAATCG (reverse primer). The cycling conditions were the same as *ESR-1* with the exception of the annealing temperature which was 66^{o}C. The total number of alleles (methylated and unmethylated) present in the amplified pool were determined using a semi-nested PCR with AGTAGTTATGGTGTTGTGGGAGTTT as forward primer and CAAAATATCCAACTACTACACTACC as reverse primer. Again the cycling conditions were the same as *ESR-1* with the exception of the annealing temperature which was 60 ^{o}C.

3.7 GENE EXPRESSION – MICROARRAY STUDIES

3.7.1 OVERVIEW

The analysis of gene expression was performed using Agilent's rat cDNA microarray (Agilent Technologies, Palo Alto, CA, USA). This technology allowed us compare differential levels of gene expression of~~in~~ 14500 separate genes in the distal oesophagus of the experimental rats at different time points after surgery with age matched non-operated controls. By competitively hybridising differentially labelled cDNA targets synthesised from both the experimental and control rats to a single rat array any difference in the level of gene expression would be detected. To make the comparison it was first necessary to convert total-RNA extracted from the tissue samples to cDNA.

3.7.2 RNA EXTRACTION

3.7.2i General

RNA extraction was performed using Q~~u~~iagen's RNeasy Mini kit (Qiagen, Crawley, UK).

To reduce the risk of RNA contamination during the procedure, all disposable equipment including scalpels, eppendorfs, pestles, and pipettes were sterilised prior to the experiment. This was achieved by autoclaving under standard conditions of 15 pounds/sq inch (1 atmosphere) above atmospheric pressure (760 mm Hg), at 121°C for in 15 minutes. The components of the RNeasy Mini kit, medicons, syringes and needles were sterilised by the manufacturer.

To prevent RNA degradation, all tissue samples were thawed slowly by placing them directly on ice after removing from the freezer, and repeated freeze-thaw episodes were avoided.

All centrifugation was performed at 25^{o}C

Solution preparation

1. B-mercaptoethanol was added to the Buffer RLT (RNeasy Mini kit) in a volume of 10µ~~u~~l B-mercaptoethanol to 1ml Buffer RLT

2. The Buffer RPE (RNeasy Mini kit) was diluted with absolute ethanol (96-100%) in a volume if 1 volume Buffer RPE to 4 volumes absolute ethanol.

3.7.2ii Release of RNA from Tissues

The frozen tissue samples stored in RNA later were transferred to an icebox and allowed to thaw. After weighing, approximately 20mg of tissue from each sample was transferred to a sterile syringe medicon used for tissue disruption. 600µ~~u~~l of Buffer RLT was added to the medicon and the tissue disrupted for 5 minutes. Tissue lysis was completed by removing the entire sample together with the Buffer RLT from the medicon using a 1ml syringe, and then repeatedly squirting out then drawing the solution up into the same syringe ~~passing the sample~~ through a 21 gauge needle for 3 minutes. The resulting tissue lysate was spun in a centrifuge at 23000g for 3 minutes, and the supernatant transferred to a sterile 2 ml eppendorf using a wide bore pipette to avoid shearing the RNA.

3.7.2iii Binding of RNA to Column

Following tissue lysis, one volume of 70% ethanol was added to the supernatant of each sample and mixed gently. The entire sample together with any precipitate was then transferred to a binding column (Rneasy Mini Column) and spun in a centrifuge at 8000g for 15 seconds.

3.7.2iv Washing the Column.

After discarding the binding column collection tube and replacing it with a new one, 700µl RW1 Buffer (kit) was added to the column using a sterile pipette. The column was then re-spun in the centrifuge at 8000g for 15 seconds.

The binding column collection tube was again discarded and replaced it with a new one. 500µl RPE Buffer (kit) was added to the binding column and spun at 8000g for 15 seconds. This procedure was repeated a second time, but the on the second occasion the column was spun at 8000g for 2 minutes to dry the column.

3.7.2v RNA Elution

A new collection tube was placed on the binding column. 30µl RNase-free water was added directly to the binding column membrane (kit), and allowed to stand for 1 minute. The binding column was then spun in the centrifuge at 8000g for 1 minute. To maximise the amount of RNA obtained from the column, this procedure was repeated but without changing the collecting tube. This resulted in approximately 60µl of RNase-free water containing purified RNA in the final sample. The extracted RNA was stored at –80^{o}C.

3.7.3 TOTAL RNA QUANTIFICATION AND QUALITY CONTROL

The concentration and quality of the extracted total RNA was determined by electrophoresis using and Agilent's RNA 6000 Nano labchip kit (Agilent Technologies, Palo Alto, CA, USA), and Agilent's 2100 bioanalyser (Agilent Technologies, Palo Alto, CA, USA). The chip contains 16 separate wells, 3 to facilitate loading of a gel onto the chip, one for a control RNA ladder, and 12 for individual RNA samples.

3.7.3i Preparation of Gel-Dye Mix

The gel was prepared by adding 550µl of RNA 6000 gel-matrix to a spin filter (kit), and spinning in a centrifuge at 1500g for 10 minutes at room temperature. The filtered gel was then transferred to 0.5ml sterile RNAse-free eppendorfs in aliquots of 65µl. The filtered gel was stored at 4ºC and used within 4 weeks of preparation.

The RNA 6000 Nano dye concentrate was allowed to warm to room temperature for 30 minutes. After mixing the dye on a vortex for 10 seconds, 1µl of dye was added to a 65µl aliquot of the filtered gel, and the gel-dye solution again mixed on a vortex for 10 seconds. The gel-dye mix was then spun in a centrifuge at 13000g for 10 minutes at room temperature. All gel-dye mix was used on the same day it was prepared.

3.7.3ii Preparation of Nano Labchip

The Nano chip was prepared using the chip priming station (kit) which facilitates distribution of the gel-dye mix on the chip. The chip was placed in

the priming station, and 9µl of gel-dye mix added to the appropriate gel introduction well, marked **G** on the chip. Using a 1ml syringe attached to the priming station, the plunger was withdrawn to 1ml, and the priming station closed. The syringe plunger was then depressed until it clipped onto the priming station. After 30 seconds the clip was released and the syringe plunger slowly pulled back to the 1ml position. A further 9µl of gel dye mix was then added to two additional gel wells marked **g**. After loading the gel, 5µl of RNA 6000 Nano marker was added to all sample wells on the chip, and the well for the RNA ladder.

The samples of RNA for analysis were loaded by adding 1µl of each sample into each of the 12 sample wells. Each RNA sample was run in triplicate. If fewer than 12 samples were to be analysed, an additional 1µl of Nano marker was added to the unused sample wells. In addition, 1µl of RNA ladder (kit) was added to the well for the RNA ladder. Once the samples were loaded, the chip was placed horizontally in the adapter of the IKA vortexer, and mixed at 2400rpm for 1 minute.

Once loaded, the chip was run on the Agilent 2100 bioanalyser.

3.7.3iii Running the Chip

Agilent's 2100 bioanalyser detects micromolecules by laser-induced fluorescence after separation by electrophoresis. The fluorescent dye in the gel-dye mix intercalates directly with RNA facilitating their detection.

Features of good quality total RNA when assessed using the bioanalyser include two distinct narrow bands or peaks corresponding to 18s and 28s ribosomal RNA at approximately 42 seconds and 48 seconds respectively, and a 28s to 18s ribosomal RNA ratio of 2. Indications that the total RNA is of poor quality include the broad based ribosomal RNA peaks with a reduction in the relative height of the peaks, and the presence of additional peaks in the baseline between the two ribosomal RNA peaks. An example of a virtual gel image and a bioanalyser trace for a sample of good quality total RNA is shown in figure 11.

3.7.4 RNA PREPARATION AND LABELLING

3.7.4i General

This was performed using Agilent's Fluorescent Direct label kit (Agilent Technologies, Palo Alto, CA, USA).

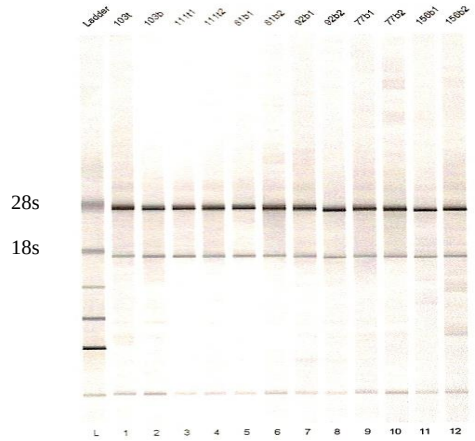
All disposable equipment including pipette tips and eppendorfs were sterile and nuclease-free. This was achieved by autoclaving the disposable equipment immediately prior to the experiment. The disposable equipment and reagents in the Direct labelling kit had been sterilised by the manufacturer. To prevent contamination during the experiment, gloves were worn at all times.

3.7.4ii Synthesis of Labelled cDNA

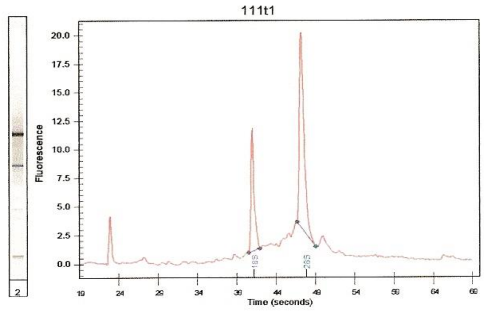
Having determined the concentration of RNA extracted from each sample, 10µg of total RNA was added to a sterile nuclease-free 2ml eppendorf. 1µl of DNA Primer was added to the RNA, and the total sample volume made up to

25µ~~l~~l with nuclease free water. The sample was incubated at 70°C for 10 minutes to

A



B



Corrected RNA Area: 92.89 rRNA Ratio [28S / 18S]: 1.97
RNA Concentration: 119.49 ng/ul

#	Name	Start Time (s)	End Time (s)	Corr.Area	% of total Area
1	18S	40.55	41.95	12.77	13.75
2	28S	46.75	49.05	25.17	27.10

Figure 11. (**A**) Good quality total RNA demonstrated on a virtual gel obtained from the bioanalyser. Two distinct bands can be seen for each of the total RNA samples analysed with no major additional bands present. The intensity of the 28s RNA band is twice that of the 18s RNA band. (**B**) Bioanalyser trace showing good quality RNA. There are 2 crisp distinct peaks at approximately 42 seconds and 48 seconds corresponding to 18s and 28s ribosomal RNA respectively. The base line between these two peaks is flat.

denature the primer and the template RNA. The sample was then cooled on ice for 5 minutes. Once cooled, 1.25ul of either cyanine 3-dCTP (control samples) or cyanine 5-dCTP (experimental samples) was added to the sample and the eppendorf wrapped in aluminium foil to prevent light exposure (both cyanine 3 and cyanine 5 are light sensitive and will degrade if left exposed).

A cDNA Master Mix solution was made up with 6µl nuclease free water, 10µl 5x first strand buffer, 5µl 0.1M DTT, 0.5µl dNTP Mix, 0.25µl 5mM dCTP, and 2µl MMLV-RT. The Master Mix was added to the sample. Labelled cDNA was synthesised by incubating the mixture at 42°C for 60 minutes in a water bath, followed by incubation at 70°C for 10 minutes on a heating block. The sample was then cooled for 5 minutes by placing on ice. Once cool, the RNA was degraded by adding 1µl RNAse I 'A' and incubating at room temperature for 30 minutes.

An overview of the labelling procedure is shown in figure 12.

3.7.4iii Purifying Labelled cDNA

Labelled cDNA samples were purified using Qiagen's QIAquick kit (Qiagen, Crawley, UK). For each array experiment, the control sample (labelled with cyanine 3) was added to the experimental sample (labelled with cyanine 5) making up a combined solution with a volume of 100µl. 500µl of Buffer PB was added to the combined solution and mixed gently. The combined sample was then transferred to a QIAquick binding column and spun in a centrifuge at 13000g for 60 seconds. After changing the collecting tube, the column was

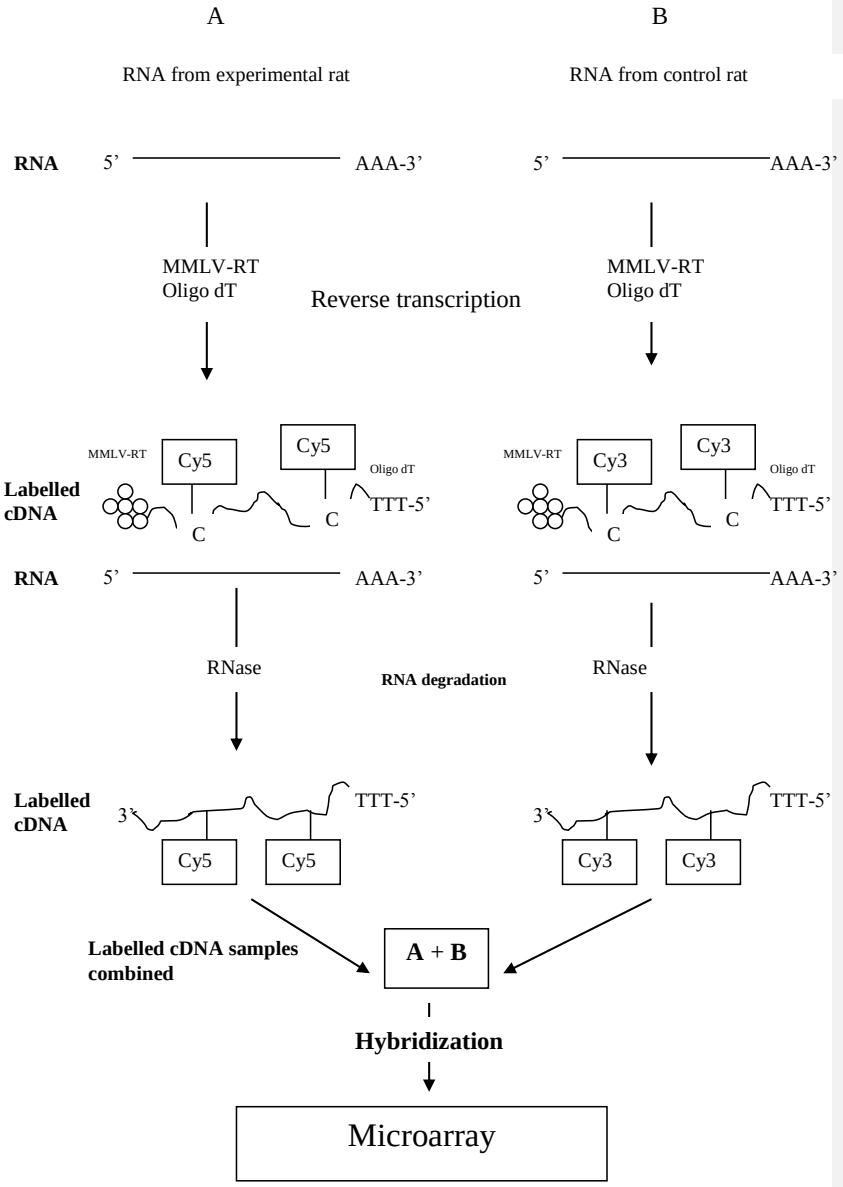


Figure 12. Overview of the cDNA labelling procedure. The experimental (A) and control (B) RNA samples are converted to fluorescently labelled cDNA by reverse transcription, and through incorporation of cyanine-5 or cyanine-3 into the experimental and control cDNA respectively. Following RNA degradation, the labelled cDNA samples are combined and hybridised to the microarray.

washed by adding 400µl of Buffer PE and spinning at 13000g for 60 seconds. This procedure was repeated for a second time following which the sample was eluted into a clean collection tube by adding 30µl of Qiagen Buffer EB directly to the column membrane and, after allowing to sit for 1 minute, spinning the column at 13000g for 60 seconds. To maximise the amount of labelled cDNA removed from the column, the elution procedure was repeated a second time.

The labelled cDNA solution was then dried in a rotary desiccator.

3.7.5 MICROARRAY HYBRIDISATION PROTOCOL

3.7.5i General

Prior to beginning the hybridisation procedure the following solutions were made up:

10x control target

A lyophilised control target pellet was added to 0.5ml sterile deionised nuclease free water. The solution was mixed for 3 seconds using a vortex.

Wash solution 1

300ml 20x SSC and 0.5ml 10% Triton X-102 was added to 700ml sterile deionised nuclease free water. After mixing, the solution was passed through a 0.2µm sterile filter and stored at room temperature.

Wash solution 2

5ml 20x SSC and 0.5ml 10% Triton X-102 was added to 995ml sterile deionised nuclease free water. After mixing, the solution was passed through a 0.2µm sterile filter and stored at 4°C.

3.7.5ii Hybridisation

The dried combined cDNA sample was re-suspended in 200µl sterile deionised nuclease free water and heat denatured by incubating at 98°C for 3 minutes on a heating block. 175µl of the cDNA sample was transferred to a new sterile deionised nuclease free eppendorf and mixed with 50µl 10x control target and 225µl 2x hybridisation buffer (kit).

To hold the hybridisation solution in contact with the microarray during the hybridisation procedure a gasket slide was placed over the array slide and held in place using a slide chamber. To achieve this, the gasket slide was first loaded into the base of the slide chamber. The hybridisation solution was transferred slowly onto a gasket slide avoiding any air bubbles, and the array slide aligned to and then placed on top of the gasket slide to form a sandwich. The slide chamber cover was placed over the sandwiched slides, and the chamber clamp assembly tightened. The entire slide chamber and array assembly was then incubated in a hybridisation oven at 60°C and 4 RPM for 17 hours.

3.7.5iii Washing the Microarray Slides

Three separate wash bowels were prepared; bowel A contained 250ml of Wash 1 at room temperature, bowel B the remainder of wash 1 at room temperature,

and bowel C wash 2 at 4^{o}C maintained by placing in an ice bucket. After incubation, the microarray slide assembly was removed from the slide chamber and quickly placed into bowel A. The microarray slide was prised off the gasket slide using blunt ended tweezers. The array slide was then transferred to wash bowel B for 10 minutes. The wash solution was agitated with a stirrer. Finally the array slide was transferred to wash bowel C for 5 minutes where again the wash solution was agitated using a stirrer.

Once the washing procedure was complete, the array slide were dried using a nitrogen filled airgun to blow the drops of solution off the surface of the slide. The array slide was stored in the dark at room temperature.

3.7.5iv Generating Images from the Microarrays

The arrays slides were scanned using an Agilent dual laser Microarray Scanner System (Agilent Technologies, Palo Alto, CA, USA). Features on the microarrays were extracted using Agilents Feature Extraction Software.

3.7.5v Data Analysis

Microarray analysis was performed using Genespring 5.0 software (Agilent Technologies, Palo Alto, CA, USA). Per spot and per chip intensity-dependent Lowess normalisation was applied to the data from each array. It was accepted that transcriptional dysregulation of a particular gene had occurred if expression was up- or down- regulated by no less than 1.5-fold in at least one of the three experimental rats compared to controls.

3.8 DRUG LEVEL ESTIMATION

3.8.1 GENERAL

Approximately 2ml of blood was collected from each rat after sacrifice by cardiac puncture using a 21 gauge needle and syringe, and immediately placed on ice after transfer to a 5ml eppendorf. The blood sample was centrifuged for 3 minutes at 3000g at a temperature of 4°C, following which the supernatant was removed and placed in a clean 2ml eppendorf. The plasma samples were stored at -80°C until analysis.

3.8.2 QUERCETIN

Plasma quercetin concentrations were determined by high performance liquid chromatography (HPLC) after extraction of quercetin and its active metabolites from plasma. The extraction procedure and subsequent HPLC was performed by Dr F. Dupient, a post doctorate research scientist working in the Department of Gastrointestinal Research at The Institute of Food Research, Norwich Research Park.

3.8.2i Extraction

One millilitre of plasma was transferred to a clean 5ml eppendorf, and 10µl ascorbic acid (100mM), 100ul acetic acid (0.65mM), and 2.5ml acetonitrile was added to the sample. In addition, 10µl apignin (60µM) was added to act as an internal standard from which the recovery factor could be calculated. The solution was mixed using a vortex in 30 second bursts every 2 minutes for a total of ten minutes, following which it was spun in a centrifuge at 13600g for 10 minutes at 4°C. The supernatant was then evaporated under nitrogen flow.

3.8.2ii Enzyme Hydrolysis Protocol

A 0.1M KPO4 buffer solution was made up by adding 39µl KH2PO4 (0.2M), 61µl K2HPO4 (0.2M), and 100µl double deionised water. The dry extract was re-suspended in 100µl of the KPO4 buffer (0.1M, pH 7), and 15µl B glucuronidase (20u) and 15µl sulfatase (0.5u) added to the solution. The sample was mixed gently and then incubated at 37°C for 30 minutes, following which, the reaction was stopped by adding 120µl methanol to the solution. The sample was then filtered through a 0.2um PVDF filter into an HPLC vial.

3.8.2iii Whole Metabolites Protocol

The dry extract was re-suspended in 100µl of methanol and 100µl double deionised water. The sample was mixed gently and then filtered through a 0.2µm PVDF filter into an HPLC vial.

3.8.2iv High Performance Liquid Chromatography (HPLC)

The column was calibrated using both quercetin standards (0.01µM, 0.1µM, 1µM, 10µM, and 100µM/L in methanol), and apigenin standards (1µM, 10µM, and 100µM/L in methanol). Quercetin concentrations in the plasma samples were derived from the HPLC response and compared to the standard curve for this compounds peak area.

3.8.3 ASPIRIN

Plasma aspirin concentrations were measured using the Vitros 950 analyser (Ortho-Clinical Diagnostics, Johnson and Johnson, 100 Indigo Creek Drive,

Rochester, NY) by Dr Dawson, Consultant Biochemist, in the Department of Chemical Pathology at the Norfolk & Norwich University Hospital, UK.

3.9 STATISTICAL ANALYSIS

Comparison of the chemopreventive effect of aspirin and quercetin on disease initiation (pre-initiation) versus disease progression (long-term) was performed using a 2-sample test for equality of proportions with continuity correction based on the number of animals that completed the study. The *overall* chemopreventive effect of aspirin and quercetin was determined using a 2-sample test for equality of proportions with continuity correction based on the pooled results of the pre-initiation and long term studies for each of these agents. This was statistically valid given that there was no significant difference in cancer incidence with respect to timing of the treatment with either agent.

Analysis of the CpG island methylation data was performed using a 2-tailed unpaired t-test. A p value <0.05 was considered statistically significant.

The results of the micro-array studies are descriptive because of the small number of replicates in each group.