1. INTRODUCTION

1.1 OESOPHAGEAL CANCER

1.1.1 INCIDENCE

The incidence of oesophageal cancer has risen rapidly in the West over the last three decades. This increase is largely due to a startling increase in the incidence of oesophageal adenocarcinoma (OAC). The UK has seen an 8-fold increase during this time period, and OAC now accounts for the majority of oesophageal cancer cases reported. With 7.5-15 new cases per 100,000 population diagnosed annually, the UK currently has one of the highest incidences of OAC in Europe as shown in Figure 1.

1.1.2 RISK FACTORS

The reason for the sharp rise in incidence of OAC remains unclear. The single greatest risk factor for developing OAC is Barrett's oesophagus (BO), a columnar cell metaplasia that replaces the native squamous epithelium of the oesophageal mucosa (Spechler et al, 1986). BO is an acquired condition thought to occur as a result of chronic gastroesophageal reflux disease (Cameron et al 1985, Winters et al 1987, Provenzale et al 1994, Vaezi et al 1996, Langergren et al 1999). The risk of patients with BO developing OAC is estimated to be 30 to 60 times that of the general population (Cameron et al 1985, Spechler et al 1984, Van der Veen et al 1989, Drewitz et al 1997).

While there is compelling evidence for a causal relationship between gastroesophageal reflux disease and BO, it is estimated that only 5-10% of

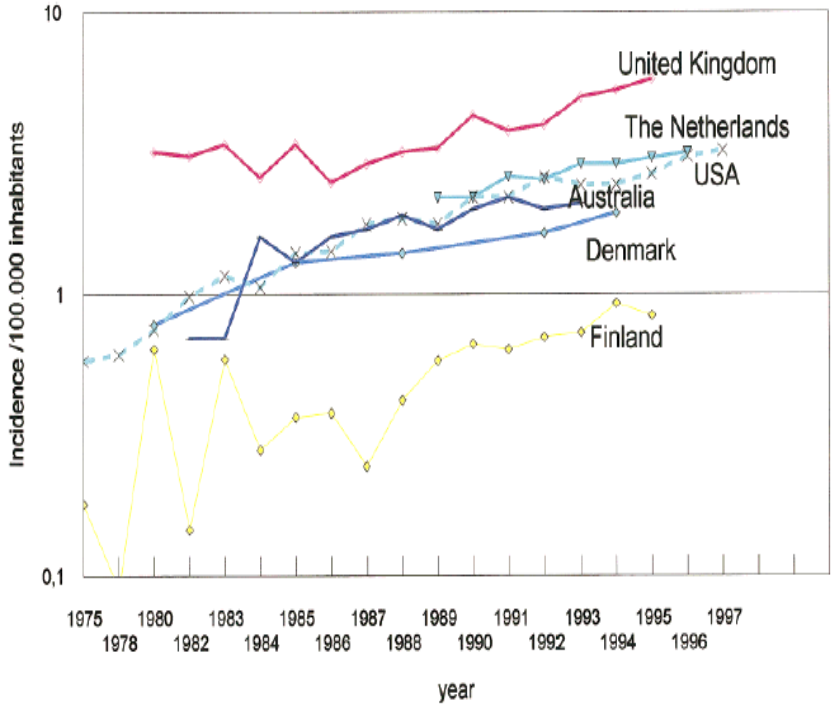

Figure 1. Incidence rates per 100,000 white males per year (age standardised to world population) of adenocarcinomas and squamous cell carcinomas of the oesophagus. Data from cancer registries represent the incidence in Australia, Europe, and the United States from 1975 to 1997

From Bollschweiler et al. Cancer 2001; 92: 549-555

patients with gastroesophageal reflux disease will develop BO (Jankowski et al 1999), suggesting additional genetic and environmental factors influence disease progression. Certainly, OAC is more common in white males (Menke-Pluymers et al 1993, Devesa et al 1998, Locke et al 1999). Evidence from a large population based case-control study in the US also identified both smoking and obesity as significant risk factors (Engel et al 2003). Smoking was associated with a population attributable risk (PAR) of developing OAC of 39.7%, while obesity was an even greater risk factor with a cumulative PAR of 41.1%. The greatest risk was observed in those with a BMI in the highest quartile. Dietary factors also appear to play an important role. Both the US study, and a second population based case-control study in Sweden (Terry et al 2001) suggest that consumption of a diet low in fruit and vegetable content is associated with an increased risk of OAC. Further analysis of theseis data specifically looking at nutrient intake suggested that dietary intake of saturated fat was significantly associated with an increased risk of OAC, and increased intake of cholesterol, animal protein, and dietary vitamin B12 were associated with an increased risk of both histological subtypes of oesophageal cancer.

1.1.3 PATHOGENESIS

The pathogenesis of Barrett's oesophagus and subsequent progression to oesophageal adenocarcinoma is not well understood. It has been proposed that Barrett's oesophagus develops in susceptible individuals in response to reflux induced damage in the distal oesophagus. It is currently believed that the columnar lined epithelium of Barrett's oesophagus arises from the native epithelial stem cells that undergo altered lines of differentiation following

chronic epithelial damage (Jankowski et al 2000). However, a recent study using an in vitro model has raised the possibility that Barretts oesophagus originates from 'transdifferentiation' of the squamous epithelium (Stairs et al 2008). This adaptive response may confer a degree of protection to the distal oesophagus to the injurious effects of the refluxate (Jankowski 1993). Continuing exposure to refluxate may result in progression to dysplasia and eventually to invasive adenocarcinoma. This stepwise model of histological progression through Barrett's metaplasia and dysplasia to frank carcinoma in response to the injurious effects of the refluxate is illustrated in Figure 2.

The contribution of the different constituents of refluxate in causing oesophageal mucosal injury and subsequent development of Barrett's oesophagus and oesophageal adenocarcinoma is poorly defined. It was initially thought that the most important noxious substances were acid and pepsin from the stomach. There is convincing evidence from both animal and human studies that acid alone, or in combination with pepsin, induces oesophageal mucosal injury (Kiliruk et al 1954, Redo et al 1959, Goldburg et al 1969, Orlando et al 1981, Hirschowitz 1991, Bremner et al 1992). It is now apparent that reflux of duodenal contents, including bile acids, also plays an important role (Vaezi et al 1996). The role of bile acids in oesophageal mucosal injury is complex. The deleterious effect appears to be dependent on both the conjugation state of the bile acids and the pH of the refluxate (Harmon et al 1981). Measurement of bile acid fractions suggests that the glycine and taurine conjugates of cholic acid are the principle bile acids in human refluxate, although unconjugated cholic acid is also present in significant amounts (Johnsson et al 1988, Iftikhar et al 1993,

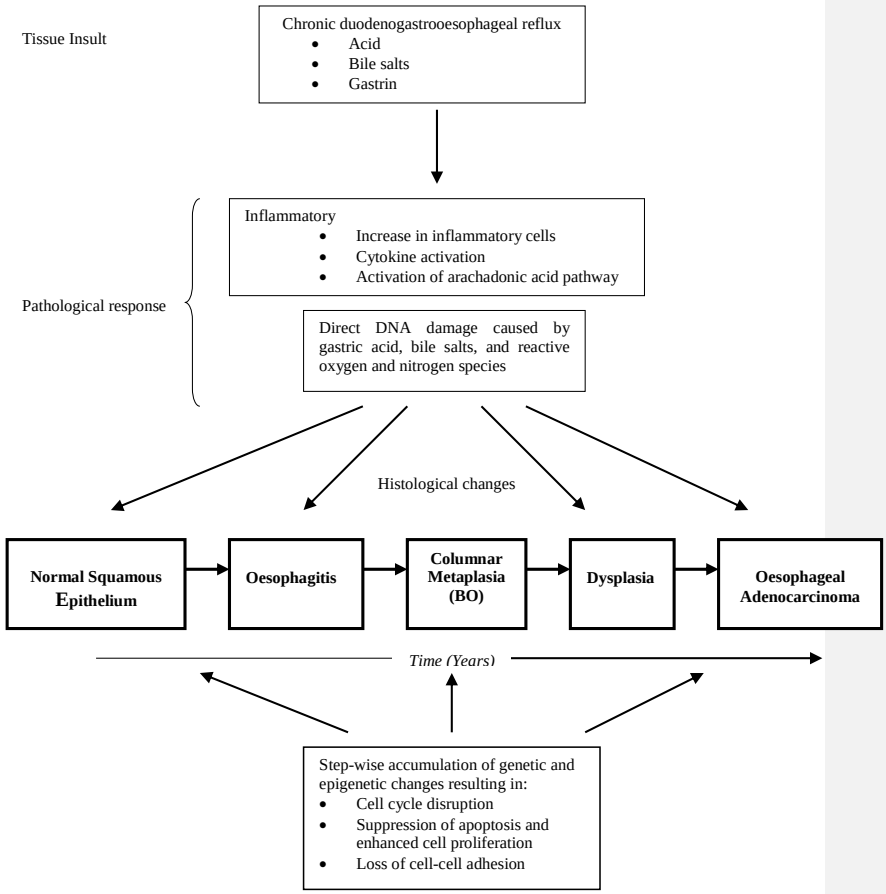

Figure 2. Summary of the histological and molecular changes that occur in the distal oesophagus in response to long term exposure to reflux of gastric and duodenal contents. As the oesophageal mucosa changes from squamous epithelium through columnar metaplasia to dysplastic mucosa there is a stepwise accumulation of genetic mutations and epigenetic events, some of which affect the mechanisms involved in the control of cell growth. Ultimately enough of these regulatory mechanisms are lost and uncontrolled cell growth occurs resulting in frank carcinoma.

Nehra et al 1999). Animal studies have shown that conjugated bile acids are more harmful in an acidic environment whereas unconjugated bile acids are more harmful at a neutral pH (Harmon et al 1981). This suggests that the bile acids present in refluxate may induce oesophageal mucosal injury over a range of pH. Vaezi and Richter demonstrated that the majority of patients with symptomatic reflux disease have simultaneous reflux of both acid and bile acids (Vaezi et al 1996). In addition, patients with combined acid and bile acid reflux have a more pronounced oesophageal injury than patients with acid reflux alone suggesting a synergistic effect, and that the severity of oesophageal injury increased with increasing concentrations of bile acids in the refluxate (Vaesi et al 1996, Oberg et al 1998, Oberg et al 2000).

The reflux induced injury to oesophageal squamous epithelium is thought to be responsible for the genetic and epigenetic changes that occur in the oesophageal mucosa which lead to the development of Barrett's oesophagus, and ultimately carcinoma. These genetic events may be induced directly by exposure to both acid and bile salts (Clemons et al 2007, Jenkins et al 2007), or indirectly through induction of reactive oxygen species (Saretzki and Von Zglinicki 2002) and nitrogen species such as nitric oxide (Clemons et al 2007, Lala and Chakraborty 2007).

1.2. MOLECULAR EVENTS IN DISEASE PROGRESSION

1.2.1 BACKGROUND

The change in phenotype of the oesophageal mucosa in response to reflux induced injury is associated with a series of genetic and epigenetic events that result in altered gene expression. These genetic events are thought to accumulate in a stepwise fashion, increasing in number as the oesophageal mucosa undergoes first metaplastic, and then dysplastic change (Jenkins et al 2002). Ultimately, transcriptional deregulation of sufficient genes involved in the control of cell proliferation occurs, and frank carcinoma develops. Transcriptional deregulation can occur directly as a result of mutations within genomic DNA (Miller et al 2003), and epigenetic events that include global genomic hypomethylation, and regional hypermethylation of CpG islands within gene coding regions of the genome (Eads et al 2001, Brock et al 2003). While still not completely understood, several studies have identified a number of genes in which deregulation of transcription appears to be important in the pathogenesis of Barrett's oesophagus and oesophageal adenocarcinoma (Pritchard et al 1994, Shirvani et al 2000, Eads et al 2001, Brock et al 2003).

1.2.2. GENETIC EVENTS

The key mutational events reported to occur in the metaplasia- dysplasia-carcinoma sequence include aneuploidy and regional chromosome abnormalities, and point mutations within individual genes. Aneuploidy is the presence of an abnormal number of chromosomes within cells. This phenomenon is strongly linked to disease progression and a predictor of adenocarcinoma development (Giaretti 1997, Barrett et al 1999). Regional

chromosome abnormalities include loss of a segment of genomic DNA, and gene amplification.

Regional chromosomal loss can result in loss of regulatory genes involved in cell proliferation. Regulatory genes commonly affected by chromosomal loss in Barrett's metaplasia and implicated in disease progression include *p16*, involved in cell cycle control and located at chromosomal position 9p21 (Galipeau et al 1999), *APC*, involved in cell adhesion and signal transduction and located at chromosomal position 5q21 (Gonzalez et al 1997), *Rb*, involved in cell cycle control and apoptosis and located at chromosomal position 13q14, *p53*, involved in DNA repair and apoptosis and located at chromosomal position 17p13 (Barrett et al 1999), and *DCC*, a cell adhesion gene located at chromosomal position 18q21 (Wu et al 1998). These chromosomal losses are also present in dysplastic tissue and adenocarcinoma ~~(Jenkins et al 2002)~~.

While not well understood, gene amplification is a process that results in extra- and intrachromosomal gains of specific DNA sequences. This process has been shown to underlie transcriptional up-regulation of a number of important genes involved in control of cell proliferation. Genes reported to undergo amplification events in Barrett's metaplasia include *TGFα*, a cell signalling gene which promotes cell division (Jankowski et al 1992). Late gene amplification events reported to occur in adenocarcinoma include the cell signalling genes *c-erbB2*, which codes for a transmembrane receptor and is located at chromosome 17q21 (Hardwick et al 1995), the *EGFR* receptor gene located at chromosome 7p12-13 (al-Kasspooles et al 1993), and the cell cycle

control genes *cyclin D1* and *cyclin E* located on chromosome 11q13 and 19q12 respectively (Kataoka et al 1999, Lin et al 2000) ~~(Jenkins et al 2002)~~.

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In addition to the effect of regional chromosomal abnormalities, point mutations within the specific DNA sequences encoding a number of key genes involved in the control of cell growth and proliferation can occur in both metaplastic, dysplastic and adenocarcinoma tissue. Many of these point mutations are associated with transcriptional deregulation and reduced levels of the gene product. Point mutations have been reported in a number of genes that are affected by regional chromosomal losses such as *p16*, *APC*, and *p53*, but may also occur in isolation.

While transcription of many important regulatory genes is deranged by defined mutational events within the genome, a number of other regulatory genes have been reported to undergo altered levels of transcription during the metaplasia-dysplasia- carcinoma sequence with no defined mutation within their coding DNA driving the change. Transcription of these genes may be altered due to loss of a gene product necessary for their transcription, an as yet undefined genomic mutation or epigenetic event.

The key cancer related genes reported to undergo altered levels of transcription during the progression of Barrett's metaplasia to adenocarcinoma, together with the genomic abnormality driving the change where known, are shown in Table 1.

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	Gene	Position	Function	Genetic abnormality		Disease stage abnormality detectable		
				Point mutation	Regional chromosomal abnormality	metaplasia	dysplasia	carcinoma
Barrett 1999	*Rb*	13q14	Cell-cycle regulation		Loss	Yes		Yes
Kataoka 1999	*Cyclin D1*		Cell-cycle regulation			Yes		Yes
Lin 2000	*Cyclin E*	19q12	Cell –cycle regulation		Gain			Yes
Galipeau 1999	*p16*	9q21	Cell-cycle regulation	yes	Loss	Yes	Yes	Yes
Hanas 1999	*p21*	6p21	Cell-cycle regulation			Yes	Yes	Yes
Wu 1998	*DCC*	18q21	Cell adhesion		Loss	Yes	Yes	Yes
Lagorce-Pages 1998	*CD44*		Cell adhesion			Yes	Yes	Yes
Gonzalez 1997	*APC*	5q21	Signal Transduction	yes	Loss	Yes	Yes	Yes
Swami 1995	*E-Cadherin*		Cell adhesion			Yes	Yes	Yes
Tselepis 2000	*P-Cadherin*		Cell adhesion					Yes
Bailey 1998	*Catenins*		Cell adhesion			Yes	Yes	Yes
Hamelin 1994	*p53*	17p13	Genome maintenance/ apoptosis	yes	Loss	Yes	Yes	Yes
Soslow 1999	*mdm2*		Genome maintenance/ apoptosis					Yes
Morales 1998	*Telomerase*		Genome maintenance/ apoptosis			Yes	Yes	Yes
Rioux-Leclercq 1999	*Bcl2*		Genome maintenance/ apoptosis			Yes	Yes	Yes
Younes 1999	*Fas*		Genome maintenance/ apoptosis				Yes	Yes
Campomenosi 1996	*k-ras*		Cell signalling				Yes (HGD)	Yes
Kumble 1997	*SRC*		Cell signalling			Yes		Yes
Jankowski 1992	*TGFα*	2p	Cell signalling		Gain	Yes		
al-Kasspooles 1993	*EGFR*	7p12-13	Cell signalling		Gain			Yes
Hardwick 1995	*c-erbB2*	17q21	Cell signalling		Gain			Yes

Table 1. Genetic abnormalities reported to occur in genes implicated in the pathogenesis of oesophageal adenocarcinoma together with the stage the abnormality is detectable along the metaplasia- dysplasia- carcinoma sequence. Genes arranged according to function ~~(Jenkins et al 2002)~~.

1.2.3 EPIGENETIC EVENTS

Hypomethylation and hypermethylation of DNA are relative terms and denote less or more methylation compared to a standard tissue. In cancer biology, the standard is the normal tissue of the organ being investigated. The cause of methylation derangement in genomic DNA during carcinogenesis in unknown, but transcriptional deregulation may occur as a consequence of this phenomenon and contribute to cancer progression.

Global hypomethylation has been reported to occur in many different types of cancer. This phenomenon can affect DNA repeats within the genome, satellite DNA, and single copies of genes, all of which have been shown to affect gene transcription (Ehrlich 2002). There is a paucity of reports on global hypomethylation in oesophageal adenocarcinoma, although it has been demonstrated in the retrotranspos~~n~~on LINE-1 (Chalitchagorn et al 2004), a highly repeated and interspersed retroviral derived DNA repeat estimated to make up approximately 15% of the human genome. DNA repeats are thought to be integral to the phenomenon of repeat-induced gene silencing during development, as they tend to be highly methylated in postnatal somatic tissues. Hypomethylation of DNA repeats can result in upregulation of their transcription, which in turn has been shown to interfere with the transcription of neighbouring genes. This phenomenon may be important in carcinogenesis.

Certain genes also undergo specific hypermethylation events as well, which usually results in transcriptional inactivation. Aberrant hypermethylation events often take place within cytosine-guanine (CpG) islands. A CpG island is a

region of genomic DNA which is rich in the dinucleotide sequence CpG. There is a lower frequency of CpG dinucleotides within genomic DNA than would be expected from base composition. The expected fraction of CpG dinucleotides is 0.4 whereas the observed frequency is approximately 0.008. This observation is thought to arise because of repeated methylation events during mammalian evolution. This involves transfer of a methyl group from S-adenosylmethionine, catalysed by DNA methyltransferase (DNMT), and results in methyl group addition at the fifth carbon position of the pyria~~a~~midine ring of the cytosine base. The addition of a methyl group to a cytosine base converts it to a thymine base. A concomitant increase in TpG and CpA dinucleotides observed within the human genome supports this theory (Bird 1980).

Despite this commonly occurring modification of genomic DNA, certain CpG islands within the genome, including regions containing genes that are involved in control of cell proliferation, are highly conserved. In particular, the promoter regions and first exon of these genes have a high concentration of preserved CpG dinucleotides (Bird 1996). Conservation of the base sequence within the promoter region is important for successful gene transcription. The mechanism by which these particular CpG islands are protected from methylation events in healthy individuals is unknown. During cancer pathogenesis, the protective mechanism is lost, resulting in methylation of these critical CpG islands. Methylation of these sites usually results in transcriptional repression (Bird et al 1999) and a reduction in the expression level of that gene. However, there are at least 3 loci where methylation activates transcription including *H19*, *Rasgrf1*,

and *Tsix* (Bell et al 2000, Hark et al 2000). Each tumour type has a characteristic set of genes with an increased propensity to become methylated.

The genes known to undergo CpG island hypermethylation in oesophageal adenocarcinoma that are implicated in cancer pathogenesis are shown in Table 2. Genes in which CpG island hypermethylation has been reported to occur early in the metaplasia- dysplasia- carcinoma sequence include *APC, p16*, *ESR*, *MGMT* and *TIMP3*. The proportion of patients with aberrant hypermethylation at these sites increases as the disease progresses to adenocarcinoma. Other genes are not affected until late in the disease process once adenocarcinoma has developed and include *CDH1* and *hMLH1* (Sato et al 2006).

Within this group of genes, CpG island hypermethylation is reported to occur most frequently in the promoter region of *APC*, hypermethylated in up to 92% of patients with oesophageal adenocarcinoma, and 82% of patients with Barrett's oesophagus. Other genes with a high frequency of CpG island methylation in include *ESR-1* which is hypermethylated in up to 86% of patients with oesophageal adenocarcinoma and 40% of patients with Barrett's metaplasia, *p16*, hypermethylated in up to 82% patients with oesophageal adenocarcinoma and 66% of patients with Barrett's metaplasia, and *TIMP3*, hypermethylated in up to 86% of patients with oesophageal adenocarcinoma and 60% of patients with Barrett's metaplasia (Sato et al 2006).

The genetic and epigenetic events implicated in carcinogenesis are not exclusive. Many of the important genes involved in the control of cell growth

Gene	Function	Normal	Metaplasia	Dysplasia	Adenocarcinoma
APC	Signal Transduction	*6.3*	*80*	*89*	*70*
p14/ARF	Cell-cycle regulation	*0*	*4*	*0*	*0*
p15/INK4B	Cell-cycle regulation	*3*	*12*	*0*	*4.5*
P16/INK4A	Cell-cycle regulation	*1.3*	*20*	*43*	*45*
HPP1	Growth factor	*3*	*44*		*68*
MGMT	DNA repair	*21*	*43*	*89*	*59*
CDH1	Cell-cycle regulation	*17*	*10*	*0*	*70*
CDH13	Cell adhesion	*0*			*14*
DAP-K	Apoptosis	*5.6*			*20*
GSTP1	DNA repair	*0*	*0*	*0*	*5*
hMLH1	DNA repair	*0*	*0*	*0*	*12*
RUNX-3	Transcription factor	*0.8*	*25*		*48*
TIMP-3	MMP/ angiogenesis	*4.8*	*60*	*78*	*56*
ESR	Cell-cycle regulation	*5*			*51*
E-Cadherin	Cell adhesion	*5*			*75*
pRb	Cell-cycle regulation	*0*			*33*
SFRP1	Signal transduction	*10*			*93*

Table 2. Genes implicated in the pathogenesis of Barrett's oesophagus and oesophageal adenocarcinoma in which CpG island hypermethylation of the promoter region has been demonstrated. The numbers represent the mean proportion (percent) of patients in which CpG island hypermethylation was observed across a number of different studies in both normal, metaplastic, and dysplastic oesophageal mucosa, as well as adenocarcinoma (Sato et al 2006, Wu et al 2007).

and proliferation such as *p16*, *APC*, and *Rb* are subject to both mutational events and methylation events during the metaplasia- dysplasia- carcinoma sequence in oesophageal adenocarcinoma. This 'double whammy' may augment transcriptional deregulation of these genes and facilitate progression to carcinoma.

1.3 MANAGEMENT

1.3.1 TREATMENT OF OAC

Surgical resection has historically been, and remains the mainstay of, curative treatment in the management of oesophageal adenocarcinoma. The aim of curative surgery is to resect the segment of oesophagus containing the tumour, together with any associated Barrett's oesophagus and the regional lymph nodes. For tumours at the gastroesophageal junction this includes the proximal portion of stomach. Curative surgical resection is offered to patients with early stage disease. This includes all patients with clinically node negative disease where the primary tumour has not breached the serosal layer of the oesophageal wall, as well as those with node negative locally advanced disease.

While alternative treatment modalities have been tested, none have been shown to be superior to surgery in patients with disease amenable to curative surgical resection. In a randomised trial comparing radiotherapy alone with radiotherapy in combination with cisplatin based chemotherapy for treatment of locally advanced (T1-3, N0-1, M0) squamous cell and adenocarinoma of the thoracic oesophagus, Cooper et al reported a 5-year survival rate of 26% in the combined therapy group. No patients in the radiotherapy group survived to 5 years (Cooper et al 1999). Following an interim analysis of the results, randomisation was discontinued, and all patients were assigned to the combined treatment group. Five-year survival of patients with both histological subtypes of oesophageal carcinoma in the non-randomised group was 14%. Subsequent subgroup analysis by histological type showed that the five-year survival for

patients with oesophageal adenocarcinoma was 12.2 %, comparable to results achieved with surgery.

However, there is some evidence that the combination of surgery and neoadjuvant chemotherapy or chemoradiotherapy for oesophageal adenocarcinoma amenable to curative surgical resection may offer a survival advantage over surgical resection alone. In a multicentre randomised trial involving 802 patients, the MRC Oesophageal Cancer Working Group demonstrated a significant survival advantage in patients with oesophageal carcinoma who received neoadjuvant cisplatin based chemotherapy prior to surgery compered to surgery alone (MRC Oesophageal Cancer Working Group 2002). The median survival of patients in the combined therapy group was 16.8 months compared to 13.3 months in the surgery alone group. While this trial included patients with both histological subtypes of oesophageal cancer, the majority (66%) had oesophageal adenocarcinoma. Concurrent use of both neoadjuvant chemotherapy and radiotherapy may offer an even greater survival advantage. In a prospective randomised controlled trial, Walsh et al reported that 32% of patients who received neoadjuvant chemoradiotherapy and surgery for oesophageal adenocarcinoma were alive at 3-years compared to only 6% of patients who had surgery alone (Walsh et al 1996). These findings concur with those reported by Urba et al in a similar randomised controlled trial who demonstrated a three year survival rate of 30% in patients receiving neoadjuvant chemoradiotherapy and surgery compared to 16% for those receiving surgery alone (Urba et al 2001).

Despite advances in surgical technique as well as the development of neoadjuvant therapies the overall 5-year survival rate for patients with oesophageal adenocarcinoma remains less than 10%. One third of patients die within one year of diagnosis, and the median survival time is 23 months. Using information collected by the Surveillance, Epidemiology, and End Results (SEER) program of the US National Cancer Institute, Farrow et al demonstrated that Stage of disease at presentation is the most important prognostic determinant (Farrow et al 1996). In this analysis of 2,986 cases of oesophageal adenocarcinoma that occurred in the US between 1973 and 1991, the overall 5-year survival rate following diagnosis was 6.8%. However, patients with in situ disease at the time of diagnosis had a 5-year survival rate of 68.2%. The 5-year survival rate decreased progressively with increasing stage of disease, and for patients with disseminated disease at the time of diagnosis was less than 2%. The overall poor survival rate reflects the fact that most patients present with disseminated disease.

1.3.2 EARLY DETECTION INITIATIVES

1.3.2i Two week wait scheme

In an attempt to facilitate early detection of cancer cases, and therefore early stage disease, the Department of Health (DoH) introduced the two-week wait scheme in the UK for a number of common cancers including oesophagogastric cancer. Under this scheme primary care practitioners were given a list of alarm symptoms and signs to help identify patients at high risk of having oesophagogastric cancer, together with improved access to specialist services. Unfortunately, despite appropriate referral of patients under this scheme the

number of oesophageal cancers detected early and therefore amenable to curative resection has not increased. Spahos et al reported that only 2 (5%) of 38 patients with upper GI cancers detected following referral under the two-week wait scheme in a large tertiary referral centre over a two-year period had early stage disease (Spahos et al 2005). This was largely because a significant proportion of patients with upper GI cancer were asymptomatic, and the alarm symptoms are only seen with late stage disease.

1.3.2ii Barrett's screening program

Given that there appears to be survival advantage if disease is diagnosed and treated at an early Stage, and that Barrett's oesophagus is the most important risk factor for developing oesophageal adenocarcinoma, many countries including the UK have introduced screening programs for patients with Barrett's oesophagus. The aim of screening is to identify patients who are either at high risk of developing oesophageal adenocarcinoma or who have frank carcinoma before the disease has disseminated. In the UK it is currently recommended that patients with non-dysplastic Barrett's oesophagus undergo endoscopic screening at 2 yearly intervals based on a risk of developing oesophageal adenocarcinoma of approximately 1% per annum. At each screening endoscopy quadrantic biopsies are taken at 2cm intervals from within the Barrett's segment, together with biopsies of any visible lesion, and examined histologically. Patients found to have low-grade dysplastic changes within the Barrett's segment are re-scoped at six monthly intervals because of the increased risk of disease progression. Surgical resection is offered to patients with either high-grade dysplastic changes within the Barrett's segment (as this is associated with a focus of

invasive carcinoma in up to 40% of patients), or low stage carcinoma (British Society of Gastroenterology 2005).

Unfortunately, despite a number of studies reporting a higher proportion of early stage cancers detected in patients undergoing Barrett's surveillance compared to those outside the surveillance program, the screening program appears to have had little impact on the overall 5-year survival rates for patients with oesophageal adenocarcinoma.

1.4. PREVENTION OF BARRETT'S RELATED OESOPHAGEAL ADENOCARCINOMA

1.4.1 INTRODUCTION

Since the prognosis for patients with OAC remains extremely poor despite improvements in conventional treatments and the introduction of the Barrett's surveillance program, attention has moved to focus on ways to prevent cancer development. Such interventions are primarily concerned with preventing progression from Barrett's metaplasia to OAC. Barrett's oesophagus is thought to arise as a consequence of chronic gastroesophageal reflux disease in susceptible individuals. Continued exposure of Barrett's metaplasia to the injurious refluxate is thought to cause progression to frank carcinoma. Abolishing the injurious effect of continued exposure to refluxate might be expected to prevent cancer development in, and possibly regression of, Barrett's metaplasia. This has been investigated both by modifying the composition of refluxate bathing the oesophageal mucosa through acid reduction, and by abolition of refluxed gastric contents into the oesophagus by surgically creating of a physical barrier at the gastroesophageal junction.

1.4.2 MODIFICATION OF REFLUXATE; THE RESPONSE TO ACID REDUCTION

1.4.2i Histamine 2 (H2) receptor antagonists.

There is no evidence that H2 receptor antagonists result in regression of Barrett's metaplasia. In a retrospective study comparing the effect of the H2 receptor antagonists cimetidine and ranitidine in a group of 35 patients under

endoscopic surveillance for long segment Barrett's oesophagus, Cooper et al were unable to demonstrate regression of Barrett's oesophagus in either group although the majority of patients reported an improvement in their reflux symptoms (Cooper et al 1987). However, this study was conducted over a relatively short period of time with the mean length of treatment in the cimetidine and ranitidine groups being 13 months and 5.7 months respectively. In a larger prospective study conducted over a longer period of time, Sampliner et al also failed to demonstrate regression of Barrett's oesophagus in a group of 76 patients with long segment Barrett's oesophagus treated with either cimetidine or ranitidine (Sampliner et al 1990). Again, the majority of patients had good symptomatic relief of reflux symptoms during treatment, but no significant reduction in the length of Barrett's segment was demonstrated during a mean follow up time of 36 months.

Similar findings were reported by Peters et al in a double blind trial in which 68 patients with long segment Barrett's oesophagus were randomised to receive ranitidine 150mg twice daily or the proton pump inhibitor omeprazole 40mg twice daily (Peters et al 1999). After 2-years of treatment, there was no change in the length of Barrett's segment in patients treated with ranitidine. However, treatment with high dose omeprazole did result in a significant reduction in the length of Barrett's segment. Despite amelioration of reflux symptoms in both groups, patients in the ranitidine group continued to have significantly increased acid reflux on pH manometry compared to those on omeprazole suggesting that incomplete acid suppression by H2 receptor antagonists may account for their failure to affect the disease process.

1.4.2ii Proton Pump Inhibitors (PPI)

Proton pump inhibitors (PPIs) are currently the preferred pharmacological agents used to suppress gastric acid secretion and control symptoms of reflux disease. However, treatment with a low dose PPI is not associated with regression of Barrett's oesophagus. In a prospective study of 24 patients treated with 20mg of omeprazole daily for up to 24 months, Neumann et al found no evidence of shortening in the length of Barrett's oesophagus (Neumann et al 1995). Fifty-four percent of patients treated with the PPI ~~did~~ developed squamous islands within the Barrett's segment, a finding not seen in a similar group of patients treated with a standard dose of H2 receptor antagonist for up to 36 months. Further evidence that low dose PPIs are ineffective at causing regression of Barrett's oesophagus was provided by the same group who in another prospective study followed 47 patients treated with 20mg of omeprazole daily for up to 6 years (Cooper et al 1998). Again no evidence of shortening in the length of Barrett's oesophagus was observed, but squamous islands within the Barrett's segment were observed in 55% of patients, mainly within 2-3 years of commencing the PPI. While no patients in either study developed carcinoma, no comment was made on disease progression from metaplasia to dysplasia within the Barrett's segment whilst receiving treatment.

The failure of low dose PPIs to cause regression of Barrett's oesophagus may be due to continued sub-clinical acid reflux. Good symptomatic control can be achieved in most patients with a low dose PPI. However, control of reflux symptoms does not necessarily equate to cessation of acid reflux. Yeh et al demonstrated that 62% of patients taking esomeprazole at a dose of 40 or 80mg

daily had significant nocturnal acid reflux despite good symptom control (Yeh et al 2003). Consequently, high doses may be necessary to completely suppress acid secretion if PPIs are to have a role in chemoprevention.

In support of this observation some studies have reported regression of Barrett's oesophagus in patients receiving high dose PPIs. Gore et al prospectively followed a cohort of 23 patients with long segment Barrett's oesophagus for 2 years after commencing treatment with 40mg omeprazole daily (Gore et al 1993). Eleven patients had previously undergone insertion of an Angelchik anti-reflux prosthesis for intractable reflux symptoms prior to the advent of PPIs. In the six months prior to commencement of omeprazole there was a small and non-significant increase in the mean length of Barrett's segment across the group. During treatment with high dose PPI a progressive and statistically significant decrease in the length of Barrett's segment was observed. In addition, squamous islands appeared within the Barrett's segment, and their number also progressively increased with duration of treatment up to 2 years.

In a follow up study of the same cohort of patients, Wilkinson et al reported no further overall decrease in the length of Barrett's segment across the group five years after commencing high dose PPI (Wilkinson et al 1999). However, the number and size of squamous islands within the Barrett's segment continued to increase with duration of treatment. One patient demonstrated complete macroscopic and microscopic regression with no intestinal metaplasia evident on biopsies taken after 4 years of treatment. In addition, one patient with low-

grade dysplasia reverted back to metaplasia after 3 years of treatment. Unfortunately, no objective measure of the reduction in acid reflux was performed during the study.

A reduction in the length of Barrett's segment during treatment with high dose PPI was also reported by Malesci et al in a prospective study of 14 patients with long segment Barrett's oesophagus treated with omeprazole 60mg once daily (Malesci et al 1996). There was a significant reduction in the length of Barrett's segment at both 6 and 12 months after starting therapy. Twenty-four hour pH monitoring demonstrated a significant reduction in oesophageal acid exposure whilst taking the PPI, reaching normal levels in 12 of the 14 patients.

Conversely, a number of prospective studies have failed to demonstrate regression of Barrett's oesophagus in patients treated with high dose PPIs. Sharma et al found that the length of Barrett's segment remained unchanged in a cohort of 13 patients with long segment Barrett's oesophagus treated with 60mg of lansoprazole daily after a mean follow up time of 5.7 years (Sharma et al 1997). Eight patients had normal pH readings~~studies~~ whilst taking the PPI. Squamous islands within the Barrett's segment were observed in 9 patients during the study.

In a larger prospective study of 188 patients, 166 with long segment Barrett's and 22 with short segment Barrett's, Cooper et al were~~as~~ also unable to show significant regression in the length of Barrett's segment despite long term PPI

therapy (Cooper et al 2006). Within the group, 144 patients were taking omeprazole at a low dose of 20mg daily, and 48 patients were taking either high

dose omeprazole or lansoprazole at a dose of 40mg or 60mg daily respectively to control symptoms. No pH studies were performed to confirm cessation of acid reflux into the oesophagus. Seventy-seven patients had taken a PPI for over 6 years, and 19 patients for over 10 years. Many patients developed squamous islands within the Barrett's segment, and the presence of squamous islands was significantly associated with duration but not dose of treatment. During the study 5 patients (0.62%) developed low-grade dysplasia between 2 and 7 years after commencing PPI treatment, and 1 high-grade dysplasia after 3 years. Further, 3 patients (0.31%) developed oesophageal adenocarcinoma after 3, 6.5 and 9.5 years of PPI treatment. Dysplasia and carcinoma occurred in patients treated with both low and high dose PPI.

The role of PPIs in preventing progression of Barrett's metaplasia through dysplasia to carcinoma is also ill defined. There is some evidence that treatment with PPIs, especially at high doses, is associated with a reduction in the incidence of dysplasia in patients with Barrett's oesophagus. In a retrospective analysis of 236 patients with newly diagnosed Barrett's oesophagus covering a 20-year period, El-Serag et al used a pharmacy database and patient notes to compare the effect of different acid suppressive therapies on disease progression (El-Serag et al 2004). Patients with dysplasia or carcinoma at the time of diagnosis were excluded from the study. In a total of 1170 patient years follow up, 56 patients developed dysplasia which occurred in 9 of 19 patients (47%) receiving no acid suppressive treatment, 25 of 64 (38%) patients receiving an H2 receptor antagonist, and 22 of 155 (14%) patients receiving a PPI. Multivariate analysis demonstrated the incidence of dysplasia in patients who

received a PPI after the diagnosis of BOE was significantly lower than those receiving either H2 receptor antagonists or no treatment at all. This finding was independent of patient demographics, year of diagnosis, and initial length of Barrett's segment. Furthermore, a longer duration of use of PPI was associated with lower incidence of dysplasia. However, the limitations of this retrospective study given its reliance on a pharmacy database mean the results have to be interpreted with caution.

In addition to the conflicting evidence that PPIs prevent progression of disease, their use presents additional problems in patients with Barrett's oesophagus. One potential mechanism for shortening of the Barrett's segment during treatment with a PPI is encroachment of squamous epithelium at the squamocolumnar junction. This can result in squamous epithelium over riding and covering intestinalised columnar epithelium. A similar phenomenon can also occur within the Barrett's segment where squamous islands develop. The buried columnar metaplasia is hidden and not amenable to endoscopic surveillance or biopsy, yet still at risk of malignant transformation. Further, long-term inhibition of gastric acid secretion may result in hypergastrinaemia. Gastrin has been shown to induce proliferation in Barrett's metaplasia, a mechanism which, at least in part, is mediated via COX-2 through the cholecystokinin 2 (CCK2) receptor, and may therefore act as a procarcinogen (Abdalla et al 2004).

1.4.3. ANTI-REFLUX SURGERY

Anti-reflux surgery has a number of theoretical advantages over medical acid suppressive treatment as a strategy to prevent progression to adenocarcinoma in patients with significant reflux disease. The surgical construction of a physical barrier should prevent any exposure of the oesophagus to the injurious effects of refluxate, rather than simply modifying its content and pH. In addition, complications such as hypergastrinaemia, which may drive carcinogenesis, are less likely to occur as gastric acid secretion remains intact obviating the feedback mechanisms that result in hypergastrinaemia frequently observed in patients treated with PPIs.

However, in a meta-analysis comparing the reported rates of oesophageal adenocarcinoma in patients with Barrett's oesophagus, Corey et al found no difference between patients treated with a surgical anti-reflux procedure compared to patients treated medically with acid suppression (Corey et al 2003). Thirty four randomised controlled trials and cohort studies were included in the analysis representing a cumulative follow up time of 4678 years in the surgical anti-reflux procedure group and 4906 years in the medically treated group. A subgroup of patients in the medical group treated with PPIs was also identified and this group had a cumulative follow up time of 4205.5 years. The studies included are shown in Table 3. Eighteen cancers occurred in the surgical group representing a cancer rate of 3.8 per 1000 patient years. This compared to 26 cancers in the medical treatment group, and 18 cancers in medical treated subgroup who received a PPI representing a cancer rate of 5.5 and 4.3 per 1000 patient years respectively. Following a multivariate analysis to control for

Author	Year	Surgical Antireflux Procedure			Medical Treatment		
		No. of Patients	Total patient-years	Total Cancers	No. of patients	Total patient-years	Total Cancers
Brand	1980	10	45	1	0	0	0
Wesdorp	1981	0	0	0	9	17	0
Skinner	1983	13	52	0	0	0	0
Starnes	1984	8	17.6	1	12	26.4	1
Cooper	1987	0	0	0	7	9.6	0
Ovaska	1989	12	80.4	2	10	67	1
DeMeester	1990	35	105	0	6	18	0
Sampliner	1990	9	27	0	58	174	0
Williamson	1990	26	109.2	3	0	0	0
Attwood	1992	19	57	1	26	78	1
Klinkenberg-Knol	1994	0	0	0	32	128	0
Sampliner	1994	0	0	0	27	78.3	0
Sagar	1995	56	392	1	0	0	0
Malesci	1996	0	0	0	14	168	0
McDonald	1996	113	734.5	3	0	0	0
Ortiz	1996	32	160	1	27	135	1
Drewitz	1997	0	0	0	170	807.5	9
Sharma	1997	0	0	0	32	98.4	1
Sharma	1997	0	0	0	13	74.1	0
Cooper	1998	0	0	0	47	182	0
Csendes	1998	152	1266	4	0	0	0
Katz	1998	16	76.8	0	0	0	0
Luostrarinien	1998	5	33.3	0	0	0	0
Low	1999	14	29.4	0	0	0	0
Patti	1999	38	72.96	0	8	15.36	0
Peters	1999	0	0	0	53	106	1
Weston	1999	0	0	0	108	359.1	5
Wilkinson	1999	0	0	0	12	57	0
Klinkenberg-Knol	2000	0	0	0	84	546	1
Lundell	2000	22	66	0	16	48	0
Sharma	2000	0	0	0	78	220.7	1
Chen	2001	45	180	0	0	0	0
Spechler	2001	129	1174	1	137	1452	4
Srinivasan	2001	0	0	0	9	40.5	0
Total		754	4678.3	18	918	4906.2	26

Table 3. Summary of the studies which met the inclusion criteria for the meta-analysis performed by Corey et al that compared the incidence of oesophageal adenocarcinoma development in patients with Barrett's oesophagus treated with either a surgical antireflux procedure and/or medical therapy. Surgical antireflux procedures did not provide any benefit in terms of reducing the incidence of cancer development compared to medical therapy (Corey et al 2003).

patient demographics and length of Barrett's segment no significant difference in the cancer rates between surgical and medical treatment was demonstrated.

In a second and more recent meta-analysis, Chang et al reported that anti-reflux surgery was associated with a significantly lower rate of OAC compared to medical treatment (Chang et al 2007). Twenty-five studies were included in the analysis representing a cumulative follow up time of 2939 years in the surgical group, and 3711 years in the medically treated group. When data from all included studies was pooled, the rate of oesophageal adenocarcinoma was 2.8 per 1000 patient years in the surgical group compared to 6.3 per 1000 patient years in the medically treated group. However, this analysis included a number of non-controlled case series which reported a much lower rate of cancer in surgically treated patients than that reported in both cohort and randomised controlled studies. When the non-controlled case series were excluded no significant difference was observed in the rate of carcinoma between the surgical and medically treated groups (4.8 cases per 1000 patient years versus 6.5 cases per 1000 patient years respectively).

These findings are supported by a large population based study by Ye et al (Ye et al 2001). Using the Swedish Inpatient Register for the years 1965-1997 inclusive, 66,965 patients with a diagnosis of gastroesophageal reflux disease were identified along with another group of 11,077 who had undergone a surgical anti-reflux procedure. Follow up information with regard to the incidence of oesophageal cancer in these groups was obtained through record linkage with a several national registers including the National Registry of

Causes of Death, the Swedish National Cancer Registry, and the Emigration Register. Using the rate of oesophageal cancer amongst the general population of Sweden as a reference, the standardised incidence ratio (SIR) was used to estimate the relative risk of developing of oesophageal cancer within these two groups. After excluding the first year of follow up, oesophageal adenocarcinoma occurred in 37 male patients treated medically representing a SIR of 6.3 (95% confidence interval 4.5-8.7). The risk of developing oesophageal adenocarcinoma was even greater in the surgically treated group where 16 male patients developed oesophageal adenocarcinoma representing a SIR of 14.1 (95% confidence interval 8.0-22.8). A similar increase was also observed in females.

These two large studies suggest that patients with gastroesophageal reflux disease treated surgically continue to have an increased risk of developing oesophageal adenocarcinoma, and indeed surgery offers no additional protection compared to medical treatment.

1.4.4. ALTERNATIVE INTERVENTIONS - Mucosal Ablation and Resection

Mucosal ablative techniques are relatively new treatments aimed at preventing the development of oesophageal adenocarcinoma in patients with Barrett's oesophagus. The goal of these therapies is to eradicate the Barrett's epithelium, and when combined with acid suppression, allow squamous differentiation of the pluripotent stem cells in the oesophagus and re-population with native squamous epithelium with a reduced propensity for malignant transformation. A number of different endoscopic treatment modalities have been utilised to achieve ablation of the Barrett's mucosa including thermal devices, and non-thermal methods such as photodynamic therapy (PDT). Thermal methods include the use of lasers, the argon plasma coagulator (apc), and more recently radiofrequency ablation (RFA) which result in coagulation and vaporisation of the Barrett's epithelium. Photodynamic therapy relies on the preferential uptake of a photosensitising agent by neoplastic tissues such as Barrett's oesophagus. Subsequent exposure to light of a specific wavelength results in cytotoxic activation and the release of reactive oxygen species causing tissue destruction in the 'sensitised' tissues.

A number of studies have reported regression of Barrett's metaplasia and a reduced tendency for progression to dysplasia and carcinoma in patients treated with ablative techniques. In a randomised trial comparing argon plasma coagulation with endoscopic surveillance alone, Ackroyd et al reported a

significant reduction in the area and length of Barrett's segment in patients treated with apc compared to controls (Ackroyd et al 2004). 40 patients with Barrett's metaplasia who had previously undergone fundoplication were randomised to apc or endoscopic surveillance. Patients randomised to apc received ablation therapy every four weeks until either no Barrett's epithelium was visible macroscopically or until a maximum of 6 treatments had been performed. After 1 year, all patients in the ablation group had a significant reduction in the overall length of Barrett's segment, with complete macroscopic regression of Barrett's epithelium observed in11 patients (55%). Regression of Barrett's epithelium was less marked in the control group with a partial reduction in the length of Barrett's segment observed in 11 patients (55%). Surprisingly, complete macroscopic regression was reported in 3 patients (15%) in this group. Two patients in the control group who had low-grade dysplasia on entering the trial had no evidence of dysplasia at 1-year follow up. No patient in either group developed dysplastic changes within the Barrett's segment during the study period. No major complications were reported in the apc group, however, 1 patient (5%) was observed to have buried glands under apparently normal neosquamous epithelium.

Bright et al have recently reported long-term follow up results for a subset of the same cohort of patients (Bright et al 2007). Fourteen of 20 patients (70%) originally randomised to apc treatment were reported to have a 95% or greater reduction in the surface area of Barrett's oesophagus compared to 5 of 20 patients (25%) in the endoscopic surveillance group after 5-years. Complete macroscopic and histological regression of Barrett's oesophagus was reported in

8 patients (40%) in the apc group compared to 3 patients (15%) in the surveillance group. There was also a non-significant reduction in the number of patients who developed dysplasia in the apc treated group. During the follow up period, 1 patient in the apc group developed low-grade dysplasia compared to 2 patients in the surveillance group. A further 2 patients in the surveillance group developed high-grade dysplasia, one of which was not fit for oesophagectomy and was therefore treated with apc. However, no patients in either group developed oesophageal adenocarcinoma. No early complications were reported in the apc group in this study but 2 (10%) subsequently developed oesophageal strictures requiring dilatation. In addition, 2 patients in both groups were reported to have buried glands from biopsies taken at their follow up endoscopies.

The findings of a potential preventative effect of argon beam plasma ablation on neoplastic progression are supported by Morris et al in a series of 55 patients with long segment Barrett's oesophagus who had either undergone anti-reflux surgery or were maintained on PPIs to control reflux symptoms (Morris et al 2001). Prior to treatment 9 patients had histologically confirmed low-grade dysplasia, and 9 patients high-grade dysplasia. After a mean follow up time of 38.5 months no patient with Barrett's metaplasia prior to ablation therapy had developed dysplasia. Moreover, there was complete regression of both high and low-grade dysplasia in all patients with Barrett's dysplasia prior to treatment. However, oesophageal perforation occurred in two patients during treatment, one of whom subsequently died.

Conversely, other groups have failed to demonstrate a long-term benefit in terms of Barrett's regression in patients with Barrett's oesophagus treated with apc. Mork et al prospectively followed up 25 patients with Barrett's metaplasia who had undergone apc, two of which had low-grade dysplasia, for a mean period of 30 months (Mork et al 2007). Twenty-one patients (84%) had complete regression of the Barrett's segment immediately following apc including the two with low-grade dysplasia. However, despite continued treatment with a PPI to suppress acid reflux, recurrence of Barrett's metaplasia occurred in 14 of these patients (66%) during the follow up period. As in other studies, no patients progressed to dysplasia or adenocarcinoma.

More recently, RFA has been used as a treatment modality for dysplastic BO with promising results. In a large multicentre randomised sham-controlled trial involving 127 patients with both high and low grade dysplasia, Shaheen et al reported a high rate of complete eradication of dysplasia and intestinal metaplasia in patients receiving RFA. Within the group of 84 patients randomised to RFA, complete eradication of dysplasia was achieved in 90.5% with low grade dysplasia compared to 22.7% in the control group, and in 81% with high grade dysplasia compared to 19% of controls 12 months after treatment (Shaheen et al 2009). Complete eradication of intestinal metaplasia occurred in 77.4% of the ablation group compared to 2.3% of controls, and did not appear to be dependent on the severity of dysplasia prior to treatment. In addition, a significantly higher rate of disease progression was observed in the control group (16.3%) compared to the RFA group (3.6%). RFA was generally well tolerated although 1 patient experienced an upper gastrointestinal bleed,

and two others chest pain necessitating hospital admission following treatment. Buried glands under neosquamous epithelium were reported in 5.1% of patients receiving RFA 12 months after treatment compared to 40% in controls.

Similar findings of regression of dysplasia in Barrett's oesophagus have been reported with photodynamic therapy (PDT). In a randomised trial comparing photodynamic therapy using the photosensitising agent aminolaevulinic acid (ALA) with placebo in patients with low-grade dysplasia in long segment Barrett's oesophagus, Ackroyd et al reported that 16 of 18 patients (89%) randomised to receive PDT had macroscopic regression of their Barrett's segment (Ackroyd et al 2000). Further, there was complete regression of dysplasia within treated Barrett's epithelium in all patients treated with PDT. This compared to regression in only 2 of 18 (11%) patients in the control group. Persistent low-grade dysplasia was present in 12 (67%) of the 18 controls. The initial response to PDT was maintained at 2 years. Moreover, there were no complications reported as a result the PDT, and no evidence of buried glands during follow up. However, the treatment did result in persistent streaks or patches of columnar epithelium rather than achieving complete circumferential ablation.

Further evidence supporting a role of PDT in the treatment of patients with dysplastic Barrett's epithelium is provided in a recent randomised multicentre trial which compared the effect of PDT using the photosensitising agent porfimer sodium and PPI treatment with PPI treatment alone (Overholt et al 2005). Two hundred and eight patients with high-grade dysplasia within a

segment of Barrett's metaplasia were randomised on a 2:1 basis to receive PDT and PPI or PPI alone. Complete ablation of high grade dysplasia was achieved in 106 of 138 (77%) patients in the PDT and PPI group compared to 27 of 70 patients (39%) in the PPI alone group, with the majority of patients achieving ablation within 12 months of treatment. During a mean follow up time of approximately 24 months 18 of 138 (13%) patients in PDT and PPI group developed oesophageal adenocarcinoma compared to 20 of 70 (29%) patients in the PPI alone group. Five-year follow-up of a subset of patients from the original cohort suggest that the initial beneficial effects of PDT and PPI compared to PPI alone in terms of progression to cancer are maintained (Overholt et al 2007). However, a number of complications were reported in the PDT treated group. These included photosensitivity reactions in 69% of patients of which 7% were severe, non cardiac chest pain in 20% of patients, and oesophageal stricture formation in 36% of patients of which one third required more than 10 endoscopic dilatation procedures.

Endoscopic mucosal resection is an alternative technique to mucosal ablation that has been used to treat patients with high-grade dysplasia in Barrett's oesophagus and early oesophageal adenocarcinoma. This procedure allows diagnostic tissue samples to be collected during treatment. In a prospective study of 61 patients with early oesophageal adenocarcinoma and 3 with high grade dysplasia, Ell et al reported complete remission following mucosal resection in 34 of 35 patients (97%) with lesions they categorised as low risk (lesions <20mm in diameter, macroscopically non-ulcerated, well or moderately differentiated, and limited to the mucosa), and in 17 of 29 patients (59%) with

lesions categorised as high risk (lesions >20mm, ulcerated, poorly differentiated, and invading the submucosa) (Ell et al 2000). The procedure was generally well tolerated but 8 patients (12.5%) had complications related to bleeding from the resection site. During a mean follow up time of 12 months, 6 patients (9%) developed local recurrence, and 3 patients (5%) developed a metachronous carcinoma, in the residual Barrett's segment.

While both ablative and resection therapies may have a role in the treatment of patients with high grade dysplasia and localised oesophageal adenocarcinoma who are unfit for surgery, the results with regard to inhibition of disease progression in patients with Barrett's metaplasia remain inconclusive. There is some concern that the addition of a thermal injury to Barrett's metaplasia during thermal ablation may hasten carcinogenesis although this is unproven. Just as with acid suppressive treatment, persistence of columnar metaplasia under newly formed squamous epithelium has been reported with both thermal and non-thermal ablative therapies. In addition, both techniques are associated with serious complications including oesophageal perforation, stricture formation, and in case of PDT a high proportion of patients may experience photosensitivity reactions. While endoscopic mucosal resection has an advantage over ablative techniques in that it provides a tissue sample for accurate staging and grading of disease it is associated with a high rate of bleeding complications and disease recurrence. Further, this technique leaves a residual segment of Barrett's oesophagus in situ facilitating the development of metachronous carcinomas. Until further work is done quantifying the impact these treatments have on cancer rates in long term, these techniques should not

be viewed as mainstream therapies to prevent cancer progression in patients with Barrett's metaplasia.

1.5 CHEMOPREVENTION

Chemoprevention is defined as the use of natural and/or synthetic substances to block, reverse, or retard the process of carcinogenesis. Given that prevention of progression of Barrett's metaplasia through the metaplasia-dysplasia-carcinoma sequence may be an effective approach to reducing mortality from OAC, and the perceived failure of anti-reflux treatments to achieve this goal, alternative candidate chemopreventive treatments have been sought. One potential candidate for preventative therapy is the non-steroidal anti-inflammatory group of drugs (NSAIDs). The bioflavonoids, which occur naturally in many plants, represent another group of compounds that may have chemopreventive properties for a number of different cancers including OAC.

1.5.1 NSAIDs

It is generally accepted that ingestion of NSAIDs is associated with a decreased risk of developing colorectal cancer. However, recent evidence suggests that NSAIDs may also be effective in preventing cancer in a range of other body sites including the oesophagus (Gonzalez-Perez et al 2003). In a meta-analysis of 9 published observational studies that looked for an association between aspirin and/or NSAID use and oesophageal cancer, Corley et al showed a protective effect of both aspirin and other NSAIDs for both histological subtypes of oesophageal cancer (Corley et al 2003). Patients with any exposure to either aspirin or NSAIDs demonstrated an overall 43% risk reduction in the odds of developing oesophageal cancer. This risk reduction increased to 46% with frequent use. Four studies provided effective estimates of risk reduction based on histological type of oesophageal cancer and demonstrated that any use

of aspirin or NSAID was associated with a 33% and 42% risk reduction in the odds of developing oesophageal adenocarcinoma and squamous carcinoma respectively. Subgroup analysis of the type of NSAID used in the study showed any exposure to aspirin was associated with a risk reduction of developing oesophageal cancer of 50% compared to a risk reduction of 25% with other NSAIDs. The studies included in the meta-analysis are summarised in tables 4a and 4b. However, the results do need to be interpreted with caution. While the epidemiological evidence for a chemopreventive effect of aspirin and NSAIDs in oesophageal cancer is compelling, there are inherent limitations to all of the studies included in this meta-analysis. The studies are observational and lack random allocation of patients and treatment. While adjustments have been made for confounding factors such as smoking and alcohol, not all studies adjusted for the same factors. In addition, aspirin use is associated with other healthy behaviour (Patterson et al 1998), and this may have an influence on cancer risk. There is also the possibility, suggested by one of the studies, that patients with reflux symptoms or a past history of peptic ulcer disease are less likely to use aspirin or NSAIDs.

The results of Corley's meta-analysis are consistent with the findings of a number of more recent studies which have reported a reduced risk of developing OAC associated with NSAID use. In a prospective study of patients enrolled in a Barrett's screening program in Seattle, Vaughan et al found the hazard ratio for developing OAC was 0.2 in NSAID users compared controls who had never taken NSAIDs during a mean follow up time of 65.5 months (Vaughan et al

Author	Year	No. Cases Oesophageal cancer	Case population	Comparison population	Design	Exposure
Thun	1993	145	Cancer study II	Cancer study II	Cohort	Aspirin
Funkhouser	1995	14	NHANES	NHANES	Cohort	Aspirin
Peleg	1995	122	Single hospital	Hospital database controls	Case-control	NSAID
Farrow	1998	486	Tumour registries	Population controls	Case-control	Aspirin/ NSAID
Coogan	2000	207	Multiple hospitals	Admissions for injuries/infections	Case-control	NSAID
Langman	2000	550	General practice database	General practice database	Case-control	Aspirin/ NSAID
Suleiman	2000	56	Death certificate registry	Deaths from myocardial infarct	Case-control	NSAID
Cheng	2000	74	Cancer registries in UK; women only	UK health service registries	Case-control	Aspirin
Sharp	2001	159	Cancer registries in UK; women only	UK health service registries	Case-control	Aspirin

Table 4a. Summary of the studies which met the inclusion criteria for the meta-analysis performed by Corley et al demonstrating a protective effect of aspirin and NSAIDs against oesophageal cancer.

		Odds Risk (95% CI) of developing OC	*p* value for homogeneity
Dose effect	Any use	0.57 (0.47-0.71)	0.38
	Intermittent use	0.82 (0.67-0.99)	0.43
	Frequent use	0.54 (0.43-0.67)	0.13
Histology	Adenocarcinoma	0.67 (0.51-0.87)	0.26
	Squamous carcinoma	0.58 (0.43-0.78)	0.20
Exposure	Aspirin	0.5 (0.38-0.66)	0.47
	NSAIDS	0.75 (0.54-1.0)	0.56

Table 4b. The results of the meta-analysis showing a reduction in the Odds Risk of developing OC with aspirin and NSAID use. The protective effect is more pronounced with frequent use, and aspirin appears to have a greater protective effect than other NSAIDs. The protective effect is seen in for both histological subtypes of OC.

2005). Similar findings were reported by Ranka et al in a case control study looking at NSAID exposure and OAC (Ranka et al 2006). After correcting for confounding factors such as smoking and alcohol consumption, NSAID use was associated with a reduction in risk of developing OAC of between 65% and 75%. A similar level of reduction in risk of developing OAC associated with NSAID use has also been reported in case control studies from Northern Ireland (Anderson et al 2006), and Australia (Sadeghi et al 2008).

The way in which NSAIDs inhibit carcinogenesis is not completely understood but it is clear that this effect is due, at least in part, to inhibition of the enzyme cyclo-oxygenase (COX) and in particular the COX-2 isoform. This inhibitory action occurs through competitive inhibition or blocking of the substrate binding site of COX in most cases with the exception of aspirin which irreversibly acetylates the COX enzyme. In addition, there is some evidence that aspirin may also have an effect at the transcriptional level and effect expression of a number of gene products necessary for cell proliferation (Hardwick et al 2004).

1.5.2 COX

1.5.2i Arachiadonic Acid Metabolism

Cyclooxygenase (COX) is an enzyme that catalyses the synthesis of prostaglandins (PG) and thromboxanes from arachidonic acid. This process is initiated by the release of free arachidonic acid from the cell membrane, a process catalysed by membrane phospholipases. Arachiadonic acid is subsequently converted to PGG2, which is then rapidly converted to the more stable PGH2 by COX. PGH2 is the common precursor of all other prostanoids, which are synthesised by tissue specific synthases (Vane 1971). The metabolic pathway is shown in Figure 3.

There are three known isoforms of COX. Expression of each COX isoform is regulated by different mechanisms resulting in different patterns of expression. COX-1 is constitutively expressed in most tissues and plays an important role in maintaining tissue integrity and cellular homeostatic functions by continuous generation of prostaglandin E2 and prostacyclin. (DuBois et al 1998, Warner et al 2004). In contrast, COX-2 is an inducible enzyme predominantly expressed at sites of inflammation in response to pro-inflammatory or mitogenic stimuli including cytokines, interleukins, growth factors, and tumour promoters (Jones et al 1993, DuBois et al 1994, Inoue et al 1995, Subbaramaiah et al 1996). Recent evidence suggests COX-2 is also expressed constitutively in some tissues including renal epithelium, vascular endothelium, central nervous system, ovaries, and uterus (Wallace 1999, Harris et al 2001, Cheng Y et al 2002). COX-3 is an alternately spliced variant of COX-1 expressed in the cerebral cortex and heart (Chandrasekharan et al 2002).

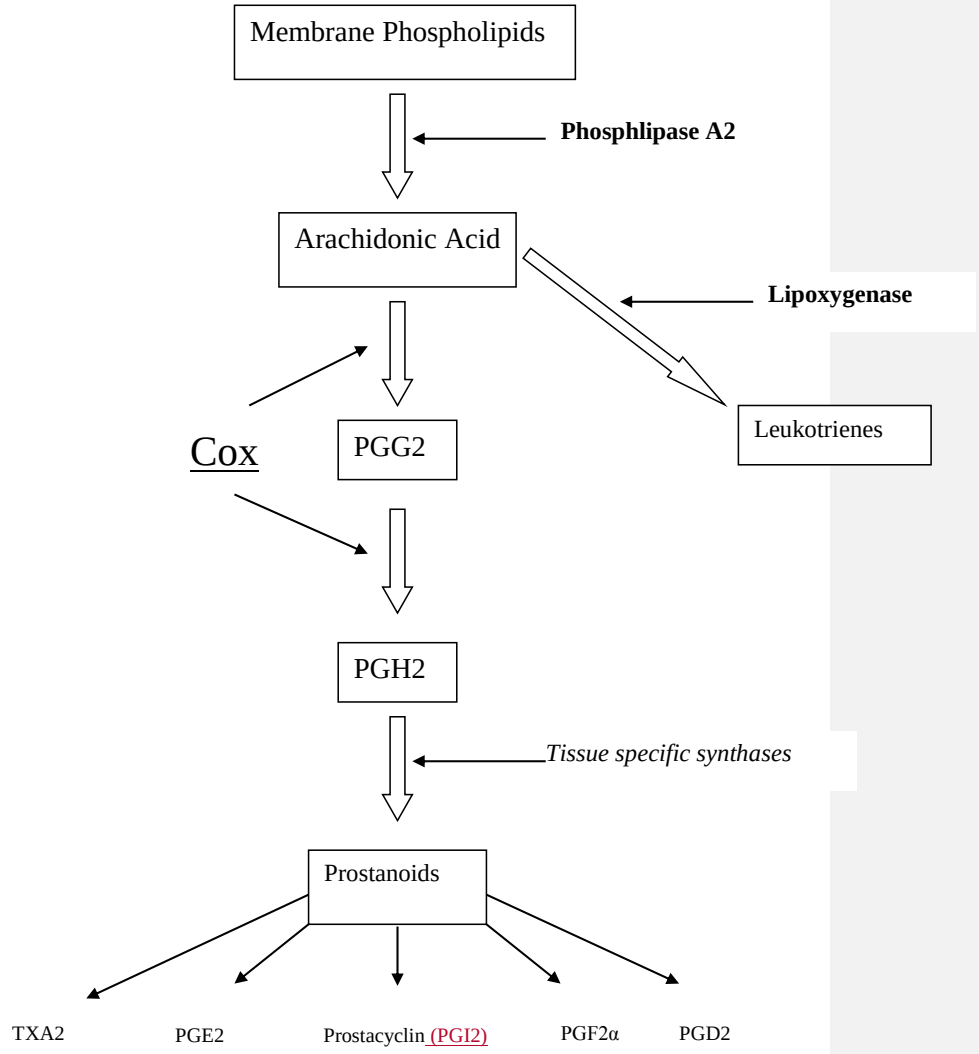


Figure 3. A flow diagram of illustrating the role of COX in arachidonic acid metabolism and prostanoid production. The prostanoid product can vary depending on the body site and circumstances of expression

1.5.2ii COX-2 and Cancer Pathogenesis

There is a substantial body of evidence linking COX-2 expression to cancer pathogenesis. Increased COX-2 expression has been reported in cancers across a range of body sites including the gastrointestinal tract. As mentioned earlier, epidemiological evidence suggests that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) may protect against the formation of gastrointestinal tumours. NSAIDs are known to inhibit cyclooxygenase. However, genetic studies provide the most direct evidence supporting a cause-and-effect connection between COX-2 and cancer pathogenesis. In a mouse model of familial adenomatous polyposis, Oshima *et al*. demonstrated that knocking out the COX-2 gene significantly reduced the number of intestinal polyps (Oshima et al 1996). Further, Lui et al. reported the development of mammary cancer in transgenic mice with forced expression of COX-2 in the mammary glands (Liu et al 2001).

The tumorogenic effect of COX-2 is thought to occur through a number of different pathways involved in tumour initiation and promotion. COX-2 promotes cell proliferation by activation of EGFR (Pai et al 2002) as well as inhibiting apoptosis, a process mediated at least in part through induction of the antiapoptotic protein Bcl-2 (Tsujii et al 1995). In addition, COX-2 has a proangiogenic effect mediated through several proangiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) (Tsujii et al 1998). A number of studies have also demonstrated that COX-2 derived prostaglandins (PGs) promote invasion and metastasis through activation of the membrane metalloproteinases 1 and 2 (MMP1 and 2) (Tsujii et

al 1997), and induction of the cell surface receptor of hyaluronate CD44 (Dohadwala et al 2001). It would also appear that COX-2 derived PGs induce immunosuppression in the local tumour microenvironment masking immune recognition and creating an environment that favours tumour growth (Plescia et al 1975).

1.5.2iii COX-2 Expression and Oesophageal Adenocarcinoma

Wilson *at al.* first reported that COX-2 was over expressed in dysplastic Barrett's oesophagus and oesophageal adenocarcinoma in 1998. In this study COX-2 mRNA expression was increased in 100% of oesophageal adenocarcinomas and 80% of non-neoplastic Barrett's oesophagus samples compared to normal gastric epithelial controls. A subsequent study by Zimmerman et al confirmed these findings with 78% of patients with oesophageal adenocarcinoma demonstrating expression of COX-2 on immunohistochemical staining (Zimmermann et al 1999). Moreover, Western blot analysis demonstrated increased expression of COX-2 but not COX-1 in the malignant tissue. Further immunohistochemical studies by Morris et al using archived tissue, and Shirvani et al who looked at endoscopic biopsies from normal squamous oesophageal mucosa, Barrett's oesophagus with and without dysplasia, and oesophageal adenocarcinoma demonstrated a progressive increase in COX-2 expression in the oesophageal mucosa through the Barrett's metaplasia-dysplasia-adenocarcinoma sequence (Morris et al 2001, Shirvani et al 2000). These~~is~~ important studies~~y~~ also demonstrated a relationship between acid and bile exposure and COX-2 expression in ex-vivo cultures of the biopsy specimens of Barrett's oesophagus. In addition, patients with oesophageal

adenocarcinoma and high levels of COX-2 expression have a poorer survival rate following surgery, and are more likely to have locoregional recurrence and distant metastases than those with lower levels of COX-2 expression (Buskens et al 2002).

1.5.2iv COX-2 and Chemoprevention

Further evidence for the functional importance of COX-2 in oesophageal carcinogenesis, and support for the role of COX-2 inhibitors as potential chemopreventive agents has been demonstrated in a number of in-vitro and in vivo studies. Souza et al demonstrated that the selective COX-2 inhibitor NS-398, but not a selective COX-1 inhibitor flurbiprofen, significantly inhibited cell growth and induced apoptosis in 2 Barrett's related OAC cell lines (Souza et al 2000). Using the same selective COX-2 inhibitor NS-398, Buttar et al showed a significant reduction in proliferation, but failed to show an associated increase in apoptosis in Barrett's epithelial cells cultured from endoscopic biopsies taken from patients with Barrett's metaplasia (Buttar et al 2002). Concurrent addition of PGE2 with NS-398 to the Barrett's epithelial cell culture negated the effect of the COX-2 inhibitor, and underlined the importance of COX-2 derived prostaglandins in oesophageal tumourogenesis. These effects were demonstrated in-vivo by Kaur et al who measured COX-2 and PGE2 levels together with PCNA expression as a marker of cell proliferation in endoscopic biopsies of Barrett's metaplasia before and after a 10-day course of the COX-2 inhibitor rofocoxib (Kaur et al 2002). Patients treated with rofocoxib had significantly reduced levels of both COX-2 and PGE2 within the Barrett's epithelium. In addition PCNA expression, which was approximately twofold

higher in Barrett's tissue compared to normal oesophagus, normalised with treatment.

In vivo studies on animal models of OAC accord with these findings and provide further evidence for COX-2 inhibitors as potential chemopreventive agents in OAC. Li et al reported a reduction in the incidence of oesophageal cancer after treatment with the novel COX-2 inhibitor JTE-522 using a rat model in which carcinogenesis was induced using the external carcinogen N-nitrosomethylbenzlamine (Li et al 2001). Interestingly, while PGE2 levels were reduced in rats receiving the COX-2 inhibitor compared to controls, levels of COX-2 were no different in either group. In another rat model of OAC in which reflux of duodenal and gastric contents was surgically induced, Buttar et al demonstrated that treatment with the selective COX-2 inhibitor MF-tricyclic significantly reduced the incidence of OAC (Buttar et al 2002). The relative risk of developing OAC in rats receiving the COX-2 inhibitor was reduced by 55% compared to controls. The reduction in cancer incidence was mirrored by a significant reduction in both COX-2 expression and PGE2 levels within the distal oesophagus of the experimental rats. More recently Oyama et al reported a significant reduction in both OAC and Barrett's oesophagus in another surgical rat model of OAC in which experimental rats received the COX-2 inhibitor celecoxib (Oyama et al 2005). As observed by Li et al, PGE2 levels were significantly reduced in rats receiving the COX-2 inhibitor compared to controls but COX-2 levels were similar in both groups. In addition there was a significant reduction in proliferative activity, and a significant increase in apoptosis in rats that received the COX-2 inhibitor compared to controls.

1.5.2v Potential Problems with NSAIDS and Selective COX-2 Inhibitors

The use of NSAIDs is associated with an increased risk of peptic ulceration and bleeding. This effect is believed to occur because NSAIDs are non-selective COX inhibitors and inhibition of COX-1, the COX isoenzyme predominantly expressed in the epithelium of the GI tract, limits the production of cytoprotective prostaglandins rendering the mucosa of the upper GI tract prone to injury from gastric acid. It was hoped that the development of the specific COX-2 inhibitors celecoxib and rofocoxib would reduce GI complications associated with NSAID use. Two subsequent studies assessing the side-effects of these selective COX-2 inhibitors have shown interesting results. The Celecoxib Long-term Arthritis Safety (CLASS) Study failed to show a significant difference in adverse GI events between celecoxib and the non-specific COX inhibitors naproxen and diclofenac (Silverstein et al 2000). Rofocoxib was shown to have an improved GI safety profile compared to naproxen in the Vioxx Gastrointestinal Outcomes Research (VIGOR) study (Bombardier et al 2000). However, this study unexpectedly also demonstrated that chronic rofocoxib use was associated with up to a fivefold increase in the incidence of cardiovascular events in elderly subjects compared to naproxen and led to its withdrawal from the market in September 2004.

1.5.3 DIET AND THE FLAVONOIDS

As mentioned earlier, 2 large population based case-control studies from the US (Engel et al 2003) and Sweden (Terry et al 2001) reported that consumption of fruits and vegetables is protective for the development of OAC in these populations. In addition, there is compelling epidemiological evidence that consumption of green tea significantly reduces the risk of developing OAC (Gao et al 1994). ~~Both f~~Fruit, vegetables and green tea are rich in flavonoids, leading to the suggestion that these compounds may be responsible for the observed protective effect, and are therefore potential chemopreventive agents for OAC.

1.5.3i The Flavonoids and Quercetin

The flavonoids are a large group of polyphenolic compounds with an almost ubiquitous distribution in the plant kingdom. They are found in most edible plants such as fruits, vegetables, cereals and nuts, and plants used in beverages such as tea. Consequently, they form an integral part of the human diet. Most of the common flavonoids ingested in the human diet exhibit a range of biological activity including both pro and antioxidant effects as well as inhibition of signalling cascades and key intracellular enzymes (Formica et al 1995). Many polyphenolic compounds have been shown to exhibit anticarcinogenic activity in a two-stage mouse skin model (Gali et al 1991).

Quercetin is a major dietary flavonoid found mainly in apples, onions and tea (Sampson et al 2002). Like all flavonoids, it possesses a three-ring structure containing a central oxygenated heterocyclic ring with two further aromatic

centres as shown in figure 4. It is consumed mainly in the form of its water-soluble glycoside and is partially absorbed from the GI tract (Hollman et al 2000). While absorbed quercetin and its metabolites are detectable in human plasma after consumption and could exert a systemic effect, it is likely that the epithelial tissues of the gastrointestinal tract are exposed to much higher concentrations of this flavonoid where it may have a biological effect locally (Halliwell et al 2000).

The ability of quercetin to inhibit cancer cell growth was noted by Suolinna et al in 1975 who demonstrated inhibition of proliferation in vitro in a number of cancer cell lines including Ehrlich ascites cells, L1210 and P-388 leukaemia cells (Soulinna et al 1975). Molnar et al reported similar results demonstrating that quercetin reduced cell proliferation of NK/Ly ascites tumour cells in vitro (Molnar et al 1981). This group went on to show that mice inoculated with the same tumour cell line lived significantly longer when treated with quercetin compared to inoculated controls suggesting that quercetin has an anticarcinogenic effect in vivo. Further evidence supporting the role of quercetin as an anticarcinogenic agent in vivo was reported by Castillo et al who demonstrated that cell growth was significantly reduced in a human squamous cell carcinoma cell line that was implanted subcutaneously in Sprague Dawley rats (Castillo et al 1989). Inhibition of cell growth in this model occurred in a dose dependant fashion.

The anticarcinogenic effects of quercetin have also been demonstrated in the gastrointestinal tract. Deschner at al reported that dietary supplementation with

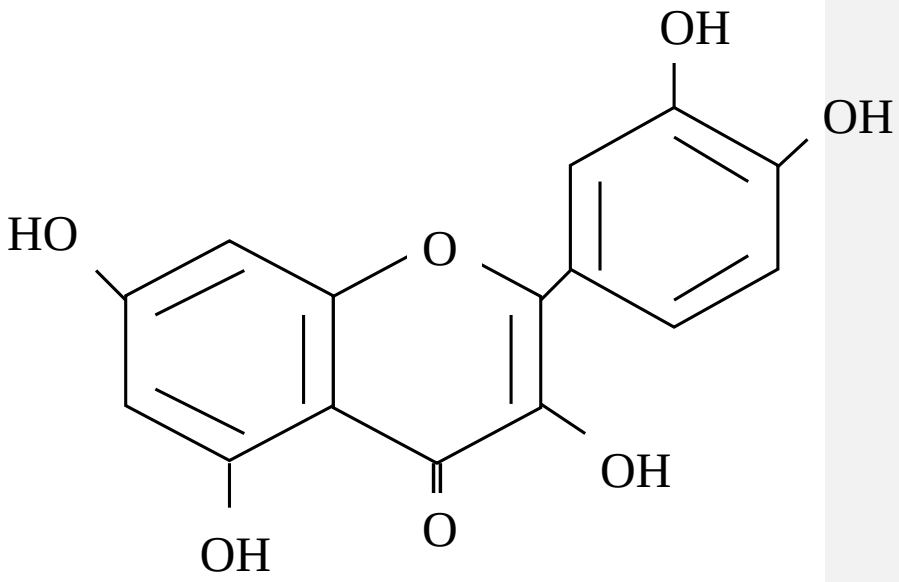


Figure 4. The chemical structure of quercetin. The three-ring structure with a central oxygenated heterocyclic ring and two further aromatic centres is common to the structure of all flavonoids

quercetin significantly reduced crypt cell proliferation, focal areas of dysplasia, and the incidence of colonic tumours in a mouse model of colorectal cancer where carcinogenesis was induced by the external carcinogen azoxymethanol (Deschner et al 1991). Using a similar animal model, Volate et al demonstrated that dietary supplementation with a number of flavonoids including quercetin significantly decreased the number of aberrant crypt foci in the colon (Volate et al 2005). In addition, the majority of the flavonoids tested induced apoptosis within the colonic mucosa with quercetin being the most potent pro-apoptotic agent. Further, Gee et al demonstrated that male Wistar rats fed a diet containing quercetin had a significantly reduced number of crypt cell mitose~~i~~s, and therefore reduced crypt cell proliferation, in both the small and large intestine compared to controls (Gee et al 2002). This group went on to show that dietary supplementation with quercetin significantly reduced the number of aberrant crypt foci in the distal colon of Wistar rats after treatment with the carcinogen 1,2-dimethylhydralazine (DMH) compared to DMH treated animals that did not receive quercetin. However, quercetin did not significantly effect the rate of apoptosis in either the large or small bowel.

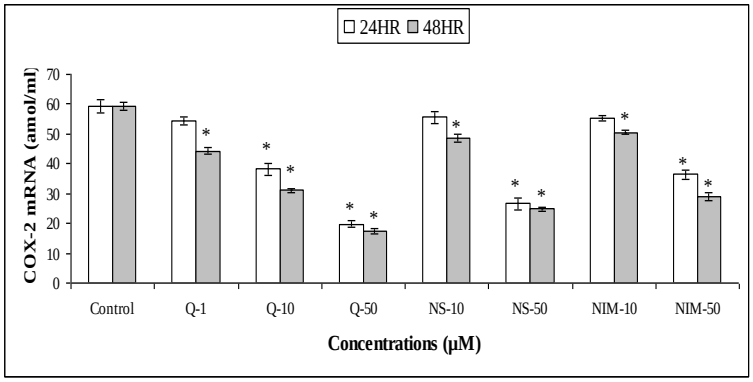
More recently quercetin has been shown to have a potent anticarcinogenic effect on oesophageal adenocarcinoma in vitro. In a comparison of the effects of quercetin with the selective COX-2 inhibitors nimesulide and NS-398, Cheong et al demonstrated that quercetin suppressed proliferation and induced both apoptosis and cell cycle arrest in OE33 cells, a Barrett's related oesophageal adenocarcinoma cell line (Cheong et al 2004). This effect was dose dependent,

and at higher doses the effects of quercetin were significantly greater than both selective COX-2 inhibitors as demonstrated in figure 5.

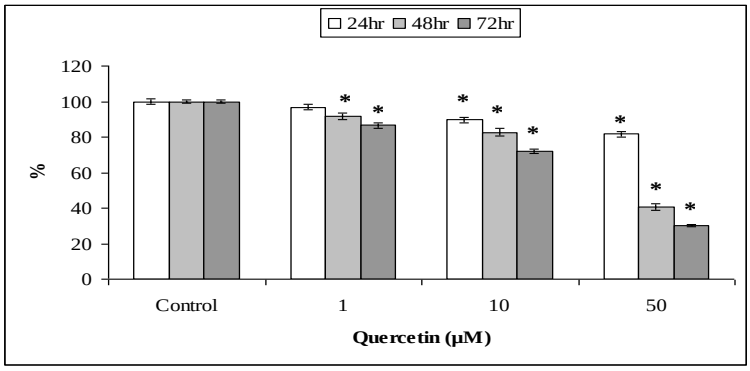
1.5.3ii Mechanism of Action

It has been proposed that quercetin inhibits carcinogenesis through a number of different mechanisms. As a powerful antioxidant, quercetin may inhibit oxidative processes implicated in cancer pathogenesis such as modification of cell transduction pathways by reactive oxygen species like the superoxide and hydroperoxyl radicals (O2-, HOO-) and reactive nitrogen species like nitric oxide (NO), as well as prevent oxidative damage to DNA by the same reactive species. Kim et al demonstrated quercetin has significant dose dependant scavenging activity against both 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 1,1-diphenyl-2-picrylhydrazyl radicals, two stable radical chromogens commonly used to assess the antioxidant capacity of compounds (Kim et al 2002). In addition, quercetin has been shown to significantly inhibit lipid peroxidation, an oxidative process implicated in carcinogenesis, induced by tert-butyl hydroperoxide in rat hepatocytes (Rodriguez et al 2001). The strong antioxidant activity appears to be due to the presence of an orthocatechol group (3'4'-OH) in the B ring (Silva et al 2002).

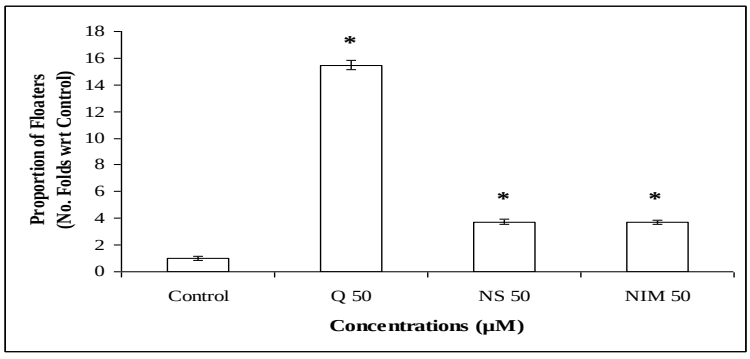
In addition to its role as an antioxidant, quercetin is an inhibitor of various protein kinases including Protein kinase C (PKC), the protein tyrosine kinase receptor epidermal growth factor receptor (EGFR), and focal adhesion kinase (FAK). Protein kinases are important enzymes involved in many intracellular signalling pathways, some of which result in transcription of genes whose



A. COX-2 mRNA expression by OE33 cells and its suppression by Quercetin and the COX-2 inhibitors NS-398 (NS) and nimesulide (NIM).



B. Inhibition of OE-33 cell growth by Quercetin.



C. Induction of apoptosis in OE33 cells by Quercetin and the COX-2 inhibitors NS-398 (NS) and nimesulide (NIM).

Figure 5. The anticarcinogenic effect of quercetin in vitro. The bioflavonoid significantly suppressed COX-2 expression (**A**), inhibited cell growth (**B**) and induced apoptosis (**C**) in the Barrett's oesophageal adenocarcinoma cell line OE33 compared to the selective COX-2 inhibitors NS-398 and nimesulide. These effects were dose dependent (*Cheong et al. Carcinogenesis 2004; 25: 1945-1952*)

products are intimately involved in the control of cell proliferation and survival. Elevated levels of expression of PTKs have been demonstrated in tumour cells. The loss of PTK regulatory mechanisms could result in uncontrolled cell growth and are implicated in carcinogenesis. Quercetin interferes with phosphorylation of these enzyme systems, possibly by competitive binding of the ATP binding site, down regulating their activity and inhibiting their ability to drive cancer progression (Kanadaswami et al 2005).

One of the products of PTK activation is COX-2, which as already mentioned is heavily implicated in the pathogenesis of many cancers including OAC. COX-2 expression is induced by stimulation of EGFR by transforming growth factor-α (TGF- α), and subsequent signal transduction by intracellular PTKs. Quercetin has been shown to be a powerful inhibitor of COX-2 expression *in-vitro* in a number of different cancer cell lines including the colon cancer cell line DLD-1 (Mutoh et al 2000), and the Barrett's related OAC cell line OE33 (Cheong et al 2004). Treatment with quercetin was also recently reported to prevent over expression of COX-2 *in-vivo* in a rat model of nephrotoxicity (Morales et al 2006). It is likely that quercetin inhibits COX-2 expression by inhibiting activation of PTKs resulting in reduced transcription of the COX-2 gene.

1.6 RAT MODELS OF OESOPHAGEAL CANCER

1.6.1 HISTORICAL DEVELOPMENT

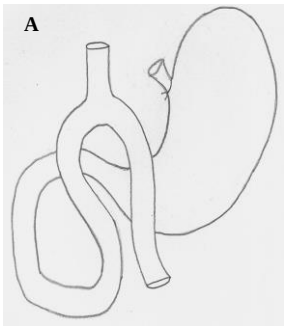
A number of rat models of oesophageal cancer have been developed over the last 4 decades to study the pathogenesis, and more recently treatment of the disease. The first models of oesophageal carcinoma used external carcinogens to induce oesophageal tumours in rats. Early experiments by Druckrey demonstrated that some asymmetric and cyclic nitrosamines induced tumour formation across a number of body sites including the oesophagus (Druckrey et al 1967). Subsequent studies confirmed these findings and showed the tumourogenic effect of nitrosamines in the oesophagus was dependent on both the dose and duration of treatment. The highest incidence of oesophageal tumours was observed in rats that receive high doses of nitrosamines given in a fractionated dosing regimen over a period of weeks (Bulay et al 1979, Siglin et al 1995). Nitrosamine compounds cause a number of histological changes in the rat oesophagus including hyperkeratosis, acanthosis, leukoplakia, leukokeratosis, and squamous cell papilloma. The carcinomas that develop are invariably squamous cell. However, when given in isolation as the only carcinogenic insult no nitrosamine compound has been shown to induce Barrett's oesophagus or oesophageal adenocarcinoma in the rat.

More recently, interest in the pathogenesis of oesophageal adenocarcinoma has led to the development of rat models of Barrett's oesophagus and oesophageal adenocarcinoma. Induction of adenocarcinoma in these models is achieved by surgically inducing reflux of gastric and/or duodenal contents into the rat

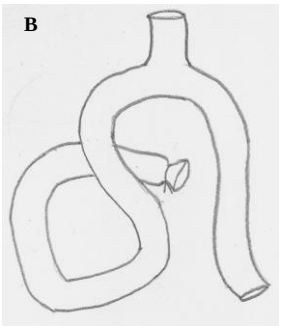
oesophagus either alone, or in combination with the administration of nitrosamine compounds.

A number of different operative techniques have been used to induce reflux of gastric and/or duodenal contents into the rat oesophagus. The majority of these operations are based on surgical models of reflux oesophagitis in the rat developed initially to determine which components of refluxate caused oesophageal injury (Helsingen 1960~~, Helsingen 1960~~, Levrat et al 1962). The different models that have been used induce oesophageal adenocarcinoma in the rat are illustrated in Figure 6.

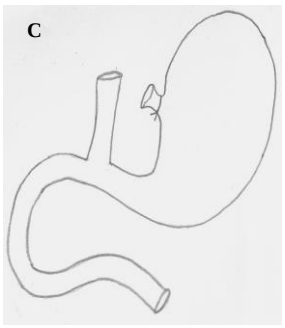
The first surgically induced oesophageal adenocarcinomas in rats were reported by Pera et al in 1989. Using a surgical model of entero-oesophageal reflux, Pera's group investigated the effect of both reflux disease induced by oesophagojejunostomy, and the nitrosamine 2,6-dimethylnitrosomorpholine (2,6-DMNM), on the oesophagus of Sprague-Dawley rats. Animals were randomised to receive surgery, 2,6-DMNM, or both. Rats that underwent oesophagojejunostomy alone did not develop oesophageal carcinoma while rats receiving only 2,6-DMNM developed oesophageal carcinomas that were invariably squamous cell in type as previously described. The combination of oesophagojejunostomy and 2,6-DMNM resulted in a higher incidence of oesophageal carcinoma compared to treatment with 2,6-DMNM alone, and both squamous cell carcinomas and adenocarcinomas were observed. The time taken to induce oesophageal cancer in these rats was significantly lower compared to rats treated with 2,6-DMNM alone. In addition, a proportion of rats that



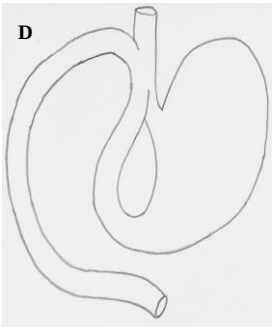
Oesophagojejunostomy (Levrat's model) *(Pera 1989, Miwa 1996)*



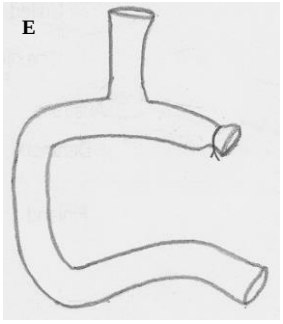
Oesophagojejunostomy + Gastrectomy *(Miwa 1996, Fein 1998)*



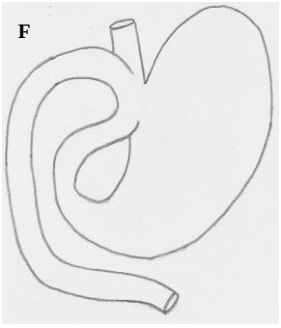
Oesophagoduodenostomy (end to side) *(Attwood 1992, Clark 1994, Goldstein 1997, Fein 1998, Oberg 2000)*



Oesophagoduodenostomy (side to side) *(Attwood 1992, Melo 1999)*



Oesophagoduodenostomy (end to side) + Gastrectomy *(Oberg 2000)*



Oesophagogastroduodenal anastomosis (EGDA) *(Chen 1999, Su 2004)*

Figure 6. Surgical models of oesophageal adenocarcinoma in the rat. The first surgically induced oesophageal adenocarcinomas were reported by Pera et al (1989) using Levrats model of enteroesophageal reflux (**A**). Subsequent models utilised either duodenal reflux (**C** and **D**) or reflux of duodenal and gastric contents (**F**) into the rat oesophagus to induced adenocarcinoma. Both enteroesophageal reflux (**B**) and duodenoesophageal reflux (**E**) can induce oesophageal adenocarcinoma in gastrectomised rats.

underwent oesophagojejunostomy developed glandular metaplasia with mucus secreting cells in the distal oesophagus whether they received 2,6-DMNM or not.

In similar studies both Attwood and Clarke confirmed these findings. Male Sprague-Dawley rats given either 2,6-DMNM (Attwood et al 1992) or methyl-n-amylnitrosamine (Clarke et al 1994) in the presence of duodenal reflux had a significantly higher rate of oesophageal cancer compared to rats given the nitrosamine compound alone, and approximately 50% of cancers observed in the combined groups were adenocarcinomas compared to 100% squamous cell carcinomas in the group treated with the nitrosamine compound alone. Importantly, in Attwoods study one rat with duodenal reflux developed adenocarcinoma in the absence of 2,6-DMNM suggesting that duodenal reflux, in the absence of an external carcinogen, can induce carcinogenesis in the rat oesophagus.

Attwood's observation that duodenal reflux per se can induce carcinogenesis in the rat oesophagus was substantiated by Miwa et al in an experiment designed to establish which fraction of refluxate was responsible for cancer induction (Miwa et al 1996). Without being exposed to an external carcinogen male Wistar rats with surgically induced reflux of either gastroduodenal or duodenal contents developed both columnar epithelium in the distal oesophagus and oesophageal carcinoma. The majority of carcinomas were adenocarcinoma. Miwa et al reported a much higher incidence of carcinoma than Attwood observed with 83% of rats with gastroduodenal reflux, and 77% of rats with duodenal reflux

developing carcinoma. However, to achieve this rats were not sacrificed until 50 weeks after surgical induction of reflux. This resulted in a high mortality rate with 60% of rats in this study dying before the end point due to non-neoplastic complications of chronic reflux such as malnutrition and pneumonia.

1.6.2 IRON SUPPLEMENTATION

One manifestation of malnutrition in rats with surgically induced reflux disease is anaemia. In most surgical models of reflux the stomach is bypassed by the formation of an oesophagoduodenostomy or oesophagojejunostomy. Anaemia is thought to develop, at least in part, due to reduced iron absorption. To correct for this Goldstein et al gave iron supplementation to male Sprague-Dawley rats following surgical induction of reflux by oesophagoduodenostomy (Goldstein et al 1997). Surprisingly, iron supplementation resulted in a higher incidence of oesophageal carcinoma within a shorter time from surgery than previously reported (Attwood et al 1992, Clarke et al 1994, Miwa et al 1996). The first cancers occurred 23 weeks after surgery, and by 31 weeks 73% of rats had oesophageal carcinoma. All carcinomas were adenocarcinoma. In addition, columnar metaplasia was observed in rats as early as 3 weeks following induction of reflux disease, and by 31 weeks 91% were affected.

Further convincing evidence that iron supplementation promotes oesophageal carcinogenesis in the rat model was provided by Chen et al in 1999. Following oesophagogastroduodenal anastomosis to induce reflux of both gastric and duodenal contents into the oesophagus, male Sprague-Dawley rats were randomised to receive either weekly iron supplementation or no iron

supplementation for the duration of the experiment. Forty weeks after surgery, rats with both reflux and iron supplementation had a significantly higher incidence of both oesophageal adenocarcinoma (53.7%) and columnar epithelium (78%) in the distal oesophagus compared to rats with reflux alone (25.6% and 53.5% respectively). Rats in a control group that received iron supplementation in the absence of reflux did not develop either oesophageal adenocarcinoma or columnar epithelium.

1.6.3 ARE OESOPHAGEAL ADENOCARCINOMAS REFLUX INDUCED?

Oberg et al raised the question of whether the development of oesophageal adenocarcinoma in the rat model was reflux-induced (Oberg et al 2000). In a study designed to determine the effect of gastric acid in the reflux model, male Sprague Dawley rats were randomly divided into 4 groups, with a different type of reflux established in each group. Three groups underwent surgery to induce reflux of both gastric and duodenal contents (oesophagoduodenostomy), duodenal contents alone (total gastrectomy and oesophagoduodenostomy), or duodenal contents in an acidic environment (total gastrectomy and oesophagoduodenostomy plus acid supplementation). The final group, which acted as controls, underwent total gastrectomy and Roux-en-Y reconstruction to prevent reflux of either gastric or duodenal contents into the oesophagus. At 24 weeks post surgery the incidence of oesophageal adenocarcinoma was similar in all groups including the controls. Approximately 80% of rats in the experimental groups had associated oesophageal inflammation compared to only 4% of controls. The authors argued this was evidence the control group did not have significant reflux disease, and therefore reflux could not be responsible for

the induction of adenocarcinoma in these rats, and was therefore unlikely to be the cause of adenocarcinoma development in those animals with reflux disease.

These results contradict all other work published on the rat surgical model, including an earlier study performed by the same group which utilised a similar study design (Fein et al 1998). In this study, male Sprague Dawley rats were randomised into 3 groups. Rats in 2 of the groups were exposed to either reflux of gastric and duodenal contents (oesophagojejunostomy), or duodenal contents alone (total gastrectomy and oesophagojejunostomy). The third group underwent total gastrectomy and Roux-en-Y reconstruction and served as control group. Rats with reflux of both gastric and duodenal contents, and duodenal contents alone had an incidence of oesophageal adenocarcinoma of 55% and 42% respectively 16 weeks after surgery. Significantly fewer rats developed oesophageal adenocarcinoma in the control group with cancers observed in only 16%. Oesophageal adenocarcinoma was only observed in rats within the control group that had inflammation of the distal oesophagus suggesting that reflux had not been completely abolished in these animals, possibly because the Roux loop was too short.

Similar findings were reported by Miwa et al in their study to determine which component of refluxate was responsible for inducing adenocarcinoma in the oesophagus (Miwa et al 1996). Male Wistar rats were randomised into 5 groups, 3 of which were exposed either to reflux of gastric and duodenal contents (oesophagojejunostomy), duodenal contents alone (total gastrectomy and oesophagojejunostomy), or gastric contents alone (transection of pylorus;

gastrojejunostomy and downstream oesophagojejunostomy with Roux-en Y reconstruction). The two remaining groups served as controls, one group undergoing total gastrectomy and Roux-en-Y reconstruction, while the other simply underwent a sham operation where the abdomen was opened and closed with no alteration in the continuity of the bowel. Fifty weeks after surgery 83% of rats with reflux of gastric and duodenal contents, and 77% with reflux of duodenal contents alone had developed oesophageal carcinoma. No cancers were observed in rats in any of the other groups including those that had undergone Roux-en-Y reconstruction.

1.6.4 DUODENAL versus GASTRIC REFLUX

The observation by Miwa et al that reflux of gastric and duodenal contents or duodenal contents alone, but not gastric contents alone induced oesophageal adenocarcinoma in the rat suggests the carcinogenic component of the refluxate is contained in duodenal juice (Miwa et al 1996). In a similar study, Melo et al exposed female Wistar rats to either reflux of gastric and duodenal contents by oesophagoduodenostomy, or gastric contents alone by cardioplasty (Melo et al 1999). Twenty-two weeks after surgery 16.7% of rats with reflux of both gastric and duodenal contents had developed both columnar metaplasia in the distal oesophagus and oesophageal adenocarcinoma. No columnar metaplasia or cancers were observed in rats with reflux of gastric contents alone.

1.6.5 IS THE RAT MODEL A GOOD MODEL OF HUMAN DISEASE?

The native epithelium of the oesophagus in the rat consists of keratinised stratified squamous epithelium that extends into the forestomach. In humans the

oesophagus is lined by non-keratinised stratified squamous epithelium which extends to the gastroeosophageal junction whereupon it changes to columnar epithelium. In addition, the rat oesophagus lacks submucosal oesophageal glands that are present in the human oesophagus. Despite these differences the histological changes reported to occur in the distal oesophagus of rats after a prolonged period of surgically induced reflux are similar to the changes reported to occur in humans. With the exception of hyperkeratosis, the rat oesophagus undergoes squamous hyperplasia and basal cell hyperplasia with severe inflammation of the entire thickness of the oesophageal wall. Ulceration and columnar metaplasia occur in a proportion of rats in the distal oesophagus proximal to the anastomosis. Tumours are invariably mucinous adenocarcinomas and develop within or adjacent to areas of columnar metaplasia in the distal oesophagus in 60-100% of animals (Pera et al 1989, Attwood et al 1992, Clarke et al 1994, Miwa et al 1996, Goldstein et al 1997, Fein et al 1998, Melo et al 1999, Chen et al 1999, Oberg et al 2000).

The progression of histological changes in response to surgically induced reflux in the rat appears to mirror human disease. In a time course study in which reflux was induced in male Sprague-Dawley rats by oesophagoduodenostomy, Goldstein et al reported that squamous hyperplasia and basal call hyperplasia were present in the distal oesophagus of all animals 3 weeks after surgery (Goldstein et al 1997). The severity and length of oesophagus affected by these changes increased with time from induction of reflux. Columnar metaplasia was present in 40% of rats at week 3, increasing to 91% by 31 weeks. The first tumour was observed at 23 weeks, and by 31 weeks 73% of rats had

histologically proven adenocarcinoma. Eighty-eight percent of tumours occurred adjacent to an area of columnar metaplasia.

Further characterisation of the rat model was provided by Su et al who examined the histological and molecular changes in the oesophagus of male Sprague-Dawley rats given iron supplementation 40 weeks after induction of chronic reflux by oesophagogastroduodenal anastomosis (Su et al 2004). Seventy percent of rats developed adenocarcinoma localised to the distal oesophagus just proximal to the anastomosis. All cancers arose from within an island of columnar metaplasia and were well-differentiated mucinous adenocarcinoma. The columnar metaplasia appeared as either a single surface layer of columnar epithelium interspersed with goblet cells and tubular mucosal glands, or as multilayered epithelium consisting of both squamous and columnar cells. The columnar epithelium observed in the oesophagus was morphologically distinct from the adjoining duodenal mucosa. Mucin staining of the columnar epithelium with alcian blue/periodic acid Schiff and high diamine/Alcian blue confirmed this distinction. The columnar cells of the oesophagus stained for neutral mucin, sialomucin, and sulfomucin whereas the columnar epithelium of the duodenal mucosa did not demonstrate any staining for sulfomucin. The mucin staining pattern of columnar metaplasia and those of adenocarcinomas arising from within that island of columnar metaplasia were similar with high quantities of sialomucin and sulfomucin and lower amounts of neutral mucin.

In addition, Su et al examined the expression levels and location within the cell of a number of molecular markers in the rat oesophagus. Immunohistochemical

analysis demonstrated similar expression profiles of CK7/CK20 in both the columnar lined oesophagus of the rat oesophagus and human Barrett's tissue. Das-1 and villin were also expressed in both the columnar metaplasia in the rat and human Barrett's tissue although different levels of expression were observed between the two. These differences were not statistically significant. Comparison of the expression profiles of CK7, CK20, and Das-1 in oesophageal adenocarcinoma revealed lower levels of expression in the rat oesophageal compared to humans, but villin expression was similar. A proportion of both rat and human cancers also expressed the tumour suppresser gene p53.

Further evidence of similarity at the molecular level between the rat model and human disease was provided by Jang et al in another time course experiment (Jang et al 2004). Chronic reflux disease was established in male Sprague-Dawley rats by oesophagoduodenal anastomosis but no iron supplementation was given. Eighty six percent of rats developed glandular metaplasia by 40 weeks after surgery but no cancers were observed. In addition to glandular metaplasia a large proportion of rats had histological evidence of squamous cell dysplasia. Immunohistochemical studies and semiquantitative Real Time Polymerase Chain Reaction (RT-PCR) demonstrated significantly higher levels of expression of both Cox-2 and prostaglandin E synthase in the lower oesophagus of experimental rats compared to controls. In addition, semiquantitative RT-PCR demonstrated increased expression of the prostaglandin E2 receptors E2, E3, and E4 the same tissue.

1.6.6 UNANSWERED QUESTIONS ABOUT THE RAT MODEL

Despite the clear need for an animal model of Barrett's metaplasia and oesophageal adenocarcinoma to further characterise the disease, identify potential therapeutic targets, and test potential chemopreventative agents in-vivo without risk to humans, no comprehensive validation of any one model has been performed. While the histological changes in the rat oesophagus in response to reflux induced injury appear to mirror human disease, the molecular events that accompany these changes have largely not been described. There is currently a great deal of interest in the genetic and epigenetic events that occur during the metaplasia-dysplasia-carcinoma sequence in humans. If the genetic and epigenetic events that occur in the rat oesophagus are similar to those seen in human disease the model would be a more powerful tool to use in future investigation of the disease and allow more confident extrapolation of results to the human condition.

1.6.7 SELECTION OF A RAT MODEL FOR THIS STUDY

When establishing a rat model of oesophageal adenocarcinoma for this study the aim was to select a model that most closely mimicked the oesophageal insult caused by refluxate in human disease. The oesophago-gastro-duodenal anastomosis (EGDA) model met these criteria. This model produces reflux of both gastric and duodenal contents into the oesophagus through an anastomosis between the oesophagogastric junction and the proximal duodenum. Given the continuity of the gastro-intestinal tract is maintained, this model may be expected to cause fewer nutritional deficiencies compared to other models. Indeed, Chen et al reported that serum iron, transferrin, albumin, and levels of

the fat soluble vitamins retinol and α- and γ-tocopherol were not significantly different in the EGDA model 40 weeks after surgery compared to non operated controls (Chen et al 1999). However, this model was associated with a high mortality and was therefore not used in this study.

The rat model finally selected for the study was a variation of Levrat's model, which produced reflux of proximal jejunal contents into the distal oesophagus through an oesophagojejunal anastomosis (Levrat et al 1962). In this model, both the vagal trunks and stomach were preserved. Consequently gastric secretions were still present in the refluxate albeit after passage through the duodenum. This model reliably induced Barrett's oesophagus and adenocarcinoma in the distal oesophagus of the rat in response to reflux of proximal enteral contents, and proved to be easier to maintain than the EGDA model. However, the pH of the refluxate in this model is alkaline (pH range 7.55-7.83), differing to that seen in humans. Further, although the stomach is maintained in this model, the continuity of the gastrointestinal tract is not, and ingested food bypasses both stomach and duodenum. Consequently this model does cause significant anaemia and other nutritional deficiencies. To compensate for this, as well as increase the tumour yield, all operated rats in this study received regular intramuscular iron injections throughout the study period.

2. AIMS

The aims of this study were:

1. To further validate Levrat's surgical model of OAC in the rat as a potential model for human disease by comparing the morphological, histological and molecular events that occur in the distal oesophageal mucosa of the rat with those described in human disease.

And

2. To assess the effect of the potential chemopreventive agents aspirin and quercetin on the development of BO and subsequent transformation to OAC in the rat model of OAC at both the histological and molecular level.