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Aspects of Barrett's Oesophagus and Oesophageal Adenocarcinoma

by

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ABSTRACT

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ASPECTS OF BARRETT'S OESOPHAGUS AND OESOPHAGEAL ADENOCARCINOMA

By James David Shutt

Barrett's oesophagus is a pre-malignant condition of the distal oesophagus in which an abnormal, intestinal-type metaplastic columnar epithelium replaces the squamous epithelium. This work explores three novel aspects.

The columnar-lined oesophagus above the oesophago-gastric junction is first identified at endoscopy. The Prague Circumferential & Maximal (C & M) classification system was developed to standardize this assessment. In this thesis I present the first live endoscopy assessment of the C & M criteria. The agreement between two endoscopists was consistent with the degree of agreement reported when the Prague C&M criteria were originally presented. However, there was a clinically significant variability in measuring the length of Barrett's oesophagus.

There is limited data on the role played by leukotrienes (LT), products of arachidonic acid metabolism by 5-lipoxygenase (5-LO), in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence progression. I studied expression of 5-LO pathway enzymes by immunohistochemistry in archival specimens and their activity by immunoassays in culture medium from whole biopsies. In human oesophageal carcinoma compared to Barrett's oesophagus or squamous biopsies, there was a significant increase in expression ($p \leq 0.001$) of both 5-LO and its activating protein FLAP, together with significantly-increased generation of both the LTB_4 and cysteinyl-LT branches of the leukotriene family ($p \leq 0.002$).

Topoisomerase IIα (Topo IIα) is a potential genetic biomarker that could differentiate diverse patient groups, or determine tailored therapy in oesophageal adenocarcinoma. I describe the study of Topo IIα protein over-expression by immunohistochemistry, and *Topo IIα* gene amplification, deletion or chromosome 17 polyploidy by fluorescence in situ hybridization. *Topo IIα* amplification was significantly associated with Topo IIα protein expression ($p=0.03$). *Topo IIα* amplification was significantly associated with both HER-2 protein expression and *HER-2* gene amplification ($p<0.01$). Tumours with chromosome 17 polyploidy ($HR=3.34$, $p=0.01$), and co-existing *Topo IIα* and *HER-2* gene amplification ($HR=2.28$, $p=0.05$) had higher risk of death than controls.

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DECLARATION OF AUTHORSHIP

I, James Shutt

declare that the thesis entitled

Aspects of Barrett's Oesophagus

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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- parts of this work have been published in abstract form:
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Signed: ……..

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Definitions and abbreviations

Abbreviations have been named in full when first mentioned, frequently used abbreviations include:

CLO	Columnar lined oesophagus
COX	Cyclooxygenase
FISH	Fluorescence *in situ* hybridization
FLAP	5-Lipoxygenase activating protein
HER-2	Human epidermal growth factor receptor 2
HGD	High grade dysplasia
IHC	Immunohistochemistry
LT	Leukotriene
LO	Lipoxygenase
LGD	Low grade dysplasia
Topo IIα	Topoisomerase II alpha

1 GENERAL INTRODUCTION

1.1 Introduction

Barrett's oesophagus is a condition in which an abnormal, intestinal-type epithelium replaces the stratified squamous epithelium that normally lines the distal oesophagus (Spechler & Goyal 1996b). There is now strong evidence that Barrett's oesophagus is an acquired condition consequent on longstanding gastro-oesophageal reflux disease (Spechler & Goyal 1996b). The most serious complication of Barrett's epithelium is the development of oesophageal carcinoma (Spechler 1996). It is of particular concern that the incidence of oesophageal adenocarcinoma is increasing more rapidly than any other solid organ cancer in Western Europe and the United States (Blot et al. 1991;Botterweck et al. 2000;El-Serag 2003;Blot 1994). Unfortunately, the five-year survival rate for Barrett's oesophageal adenocarcinoma is only 13-15% (Menke-Pluymers et al. 1992;Clark et al. 1996).

The development of adenocarcinoma in Barrett's epithelium follows a stepwise process from metaplasia through varying degrees of dysplasia to early carcinoma then eventually invasive cancer (Jankowski et al. 1999). Whilst the lifetime risk of oesophageal adenocarcinoma is increased in Barrett's, the annual incidence of high grade dysplasia and early cancer is estimated at only 0.5-1% (Spechler 2002;Jankowski et al. 2002). Most patients with Barrett's oesophagus undergo surveillance endoscopy every one to two years in an attempt to identify dysplastic changes before carcinoma develops or at least detect cancers before they disseminate to local lymph nodes, when the chance of curative surgery is greatly reduced. The endoscopic surveillance program involves a huge investment in time and money and its effectiveness has been questioned (Macdonald et al. 2000). It is presently unclear why only a fraction of patients with Barrett's progress through dysplasia to carcinoma and the factors driving this progression also remain unclear. Greater understanding of the natural course of Barrett's and the identification of factors open to therapeutic modulation could lead to a rationalized approach to endoscopic surveillance (Souza & Spechler 2005).

The definition of Barrett's oesophagus continues to evolve and remains a subject of debate in the gastroenterological literature. The absence of a universally accepted definition of Barrett's oesophagus has lead to confusion and difficulties in comparing different studies of this premalignant condition. Guidelines have been issued by the American College of Gastroenterology and by the British Society of Gastroenterology, and an American Gastroenterology Association workshop tackled the issue of defining Barrett's oesophagus (Sampliner & Practice Parameters Committee of the American College of Gastroenterology 2002;Playford 2006;Sharma et al. 2004). There is a difference of opinion between British and American pathologists regarding the identification of intestinal metaplasia to make a diagnosis of Barrett's oesophagus, but there is uniform agreement on the importance of replacement of the normal squamous lining of the oesophagus by metaplastic columnar epithelium that is visible macroscopically at endoscopy (Sampliner & Practice Parameters Committee of the American College of Gastroenterology 2002;Playford 2006).

In 1950, Norman Barrett published his account of a congenital short oesophagus with intra-thoracic gastric columnar lining, and congenital gastric heterotopia with ulceration in the oesophagus (Barrett 1950). Further evidence that this was an acquired condition prevalent in patients with gastro-oesophageal reflux was provided by Allison in 1953 (Allison & Johnstone 1953). This association was confirmed by several authors, with further evidence from an animal model that substantiated the development of a columnar lined oesophagus as a response to gastro-oesophageal reflux (Moersch et al. 1959;Hayward 1961;Bremner et al. 1970). Histologically, the columnar lined oesophagus (CLO) consists of three principal cellular types: a gastric fundic type epithelium; a junctional type epithelium; and intestinal metaplasia, characterized by the presence of goblet cells (Paull et al. 1976). The realization of the malignant potential of columnar lined oesophagus bestowed great significance on the condition and therefore its accurate diagnosis (Naef et al. 1975;Haggitt et al. 1978). It has since become apparent that the risk of developing adenocarcinoma only applies to CLO with intestinal metaplasia, and that CLO with fundic epithelium has no malignant potential

(Reid et al. 1987;Spechler & Goyal 1996a). Whilst I agree with the BSG guideline's suggestion to move from the eponymous term of Barrett's oesophagus, to the more descriptive terminology of CLO, the term Barrett's oesophagus will be used throughout this thesis given its continued widespread use (Playford 2006).

The prevalence of Barrett's oesophagus is high in the general population, although many are asymptomatic and unrecognized. A study of unselected autopsies predicted a prevalence of 0.38% in Olmsted County, MN, USA. A Swedish study that surveyed a local adult population and invited a subset for endoscopy, found a prevalence of 1.6% (Ronkainen et al. 2005). In studies that offered simultaneous upper GI endoscopy to those undergoing screening colonoscopy for colorectal cancer, a prevalence of 5.6% to 15-25% in older patients, has been documented (Rex et al. 2003;Ward et al. 2006;Gerson et al. 2002). The prevalence is higher in patients with symptoms of chronic gastro-oesophageal reflux disease, at 5-15% (Voutilainen et al. 2000;Conio et al. 2002;Lin et al. 2004;Westhoff et al. 2005). The length of Barrett's, and risk of related complications increase with the degree of acid exposure and presence and size of hiatal hernias (Avidan et al. 2002;Cameron 1999;Fass et al. 2001). However, reflux symptoms are a poor predictor of Barrett's, although symptom frequency and chronicity are better predictors that severity (Voutilainen et al. 2000;Eloubeidi & Provenzale 2001;Locke et al. 2003;Conio et al. 2002;Eisen et al. 1997). When the effects of reflux disease are controlled for, the subgroups at greater risk of Barrett's oesophagus include: Caucasian race, male sex, and advancing age (Ronkainen et al. 2005;Westhoff et al. 2005;Eloubeidi & Provenzale 2001).

The risk of both Barrett's oesophagus and oesophageal adenocarcinoma is increased with increased body mass index (BMI). Increased BMI is associated with an increase in reflux symptoms (Hampel et al. 2005), with visceral abdominal tissue 1.5 times greater than controls (El-Serag et al. 2005). Indeed, this central adiposity seems critical; a population based case-control study found that waist-to-hip ratio was the most important variable (Edelstein et al. 2007). Patients with central obesity are predisposed

to hiatal hernia, but also exhibit a different adipokine profile that may increase the risk of Barrett's and cancer (Wilson et al. 1999;Rubenstein et al. 2008;Watanabe et al. 2007). Alcohol use and smoking are at best minor factors (Gerson et al. 2002;Edelstein et al. 2007). It has been postulated that *Helicobacter pylori*, in causing an atrophic gastritis, reduces gastric acid output and is protective against the development of Barrett's oesophagus. The reduction in prevalence of *H pylori* in the West has occurred during a period when gastro-oesophageal reflux disease and Barrett's has increased, but a causal role has not been established (El-Serag & Sonnenberg 1998). It is not clear why some patients develop severe oesophagitis, and others Barrett's oesophagus. It has been proposed that the cellular changes of Barrett's are a short-term adaption to reduce bleeding and stricturing (Shaheen & Richter 2009). There is no common pattern to any inheritable tendency to Barrett's oesophagus, suggesting a polygenic trait; many polymorphisms in genes involved in the inflammatory response have been described.

Endoscopy is the major diagnostic modality; commonly patients have received a therapeutic trial of acid-suppressing medication, most often a proton pump inhibitor (PPI), unless there are worrying symptoms suggesting an oesophageal malignancy. This PPI therapy could potentially reduce the recognized risk of missing shorter segments of Barrett's if oesophagitis is present (Hanna et al. 2006). Until recently, to avoid confusion between Barrett's oesophagus and the normal junctional columnar epithelium, and the difficulty inherent in identifying the oesophago-gastric junction in hiatus hernia, an arbitrary minimal length of 3cm of CLO from the oesophago-gastric junction was required before making the diagnosis of Barrett's oesophagus (Skinner et al. 1983). Since then it has become apparent that so-called "short segment" Barrett's oesophagus, lengths of CLO less than 3cm long and even short, non-circumferential tongues of columnarisation, can be associated with the development of adenocarcinoma (Schnell et al. 1992). Further studies have shown that these patients have gastro-oesophageal reflux disease, with a severity between that of patients with erosive oesophagitis and those with "traditional Barrett's oesophagus" (Clark et al. 1997). To further complicate matters, microscopic intestinal metaplasia occurring at an

endoscopically-normal oesophago-gastric junction has been described. Confusingly, this has been termed “ultra-short segment Barrett’s oesophagus”, although it appears to be associated with advancing age and Helicobacter pylori infection rather than gastro-oesophageal reflux disease, and its risk of malignant change appears to be extremely low (Spechler & Goyal 1996a;Spechler et al. 1994;Trudgill et al. 1997;Nandurkar et al. 1997;Weston et al. 1996). It has been proposed that this condition should be termed intestinal metaplasia at the cardia, with the recommendation not to biopsy a normal-appearing oesophago-gastric junction (Playford 2006).

With the healthcare costs becoming an increasing concern, and the lack of robust evidence to support the current endoscopic surveillance program (Sharma et al. 2004), there has long been a call for better diagnostic methods for Barrett’s oesophagus (Sharma & Sidorenko 2005). A key component of this will be the identification of biomarkers to identify those patients at greatest risk of dysplasia and adenocarcinoma and therefore focus surveillance, with the additional benefit of identifying novel targets for chemoprevention and therapy (McManus et al. 2004;Nicholson & Jankowski 2009).

1.1.1 Endoscopic classification of Barrett’s oesophagus

A key component of all current proposed definitions is the endoscopic identification of any length of columnar-lined oesophagus above the oesophago-gastric junction (Playford 2006;Sampliner & Practice Parameters Committee of the American College of Gastroenterology 2002). Endoscopy is a widely available, invasive diagnostic and therapeutic modality, employing flexible, controllable endoscopes to examine the gastrointestinal tract. The endoscope is attached to a light source and image processor, with images displayed on a monitor. The endoscopist uses the left hand to support the endoscope and steer the tip with two control wheels, the right hand holds the shaft near the mouth. The shaft is advanced with gentle pressure into the mouth, intubating the oesophagus by passing the cricopharyngeus (push endoscopy). The

stomach is entered with gentle pressure through the oesophago-gastric junction, which can be inspected in retroflexed position (tip deflected 180º). On withdrawal of the endoscope, the right hand is used to gently pull the endoscope out (pull endoscopy).

The most popular anatomical definitions of the oesophago-gastric junction include the proximal limit of the longitudinal gastric folds (Johnson et al. 1996;Nandurkar & Talley 1999;Tytgat 1997), the distal limit of the longitudinal oesophageal blood vessels, and the point of flaring from the tubular oesophagus into the more dilated stomach (Spechler et al. 1994), in the absence of air insufflation. Panels of experts in both America and Europe have independently agreed to use the proximal limit of the longitudinal gastric folds as a definition of the oesophago-gastric junction (Sharma et al. 2004;Brien et al. 2000), although there are scant data to validate it. The proximal margin of a segment, the squamo-columnar junction or Z-line, is more readily recognized as the transition from oesophageal mucosa to columnar lined, gastric-appearing mucosa (Figure 1.1). The length of a segment of columnar lined oesophagus is thus the difference between these anatomical landmarks, with measurements taken in centimeters from the incisors at endoscopy. At endoscopy with a living, moving oesophagus, identification and measurement of these landmarks, notably the precise oesophago-gastric junction, can prove difficult, especially in the presence of a hiatus hernia. Endoscopic criteria for Barrett's oesophagus are fairly reproducible (Sampliner et al. 1990), although diagnostic inconsistencies do occur (Kim et al. 1994).

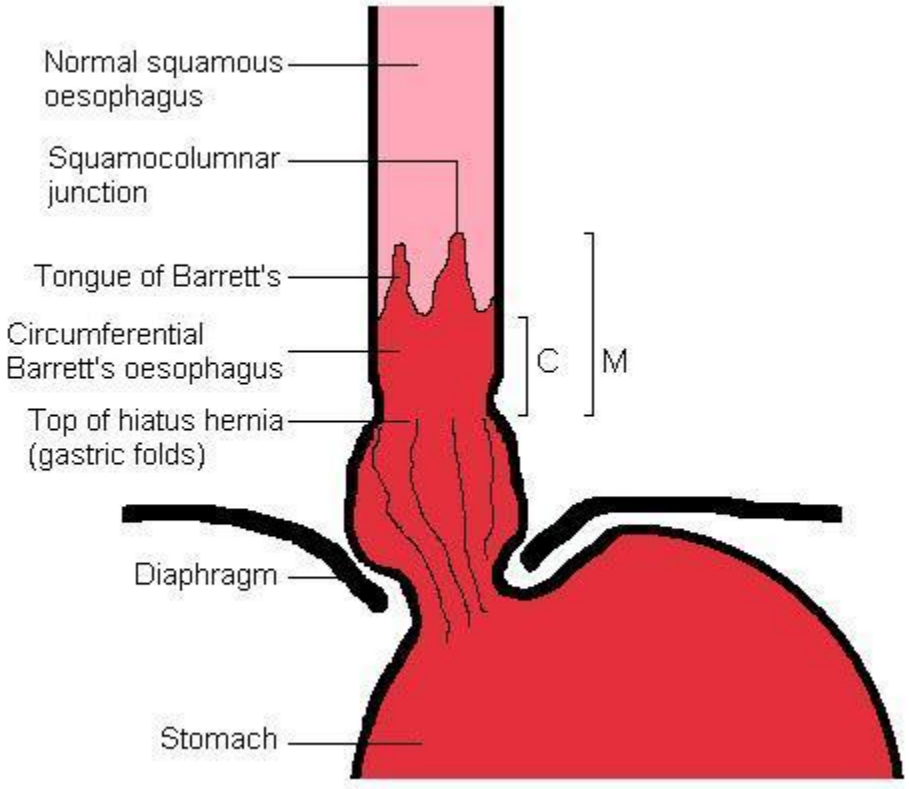
At the Digestive Diseases Week (DDW) conference in 2006, Dr P Sharma on behalf of the International Working Group for the Classification of Oesophagitis introduced a new endoscopic classification for the reporting of Barrett's oesophagus; the Prague C&M classification (Sharma et al. 2006). This represented the first step towards a standardized reporting process, allowing improved case comparison and recruitment into trials, and was modeled on the successful process that lead to the Los Angeles classification system for the endoscopic assessment of oesophagitis (Armstrong et al.

1996;Lundell et al. 1999). The classification system aims to provide clarity by describing the length of the Circumferential confluent columnar lined oesophagus, the "C" value, and the length of the Maximum extent of the columnar lined oesophagus, i.e. including tongues of columnar lined oesophagus, the "M" value. The validation data presented consisted of the inter-observer agreement between 29 independent assessors who watched 29 endoscopic videos of cases of Barrett's oesophagus on slow withdrawal of the scope after gastric deflation, with a superimposed distance marker displayed in the top left hand corner of the screen. After an introduction to the new classification, assessors were asked to use the C&M criteria on the 29 cases they witnessed. An initial pilot, involving five assessors, comparing videos of endoscope insertion and withdrawal demonstrated no appreciable difference in measurements.

The reliability coefficient (intraclass correlation coefficient) for "C" was 0.94 (95% CI: 0.91-0.97) and "M" was 0.93 (95% CI: 0.89-0.96). The overall kappa coefficient for Barrett's longer than 1 cm was 0.72, falling to 0.21 for Barrett's shorter than 1cm, resulting in a kappa of 0.49 for any length. The reliability coefficients for anatomical landmarks were very high, 0.88 for the proximal margin of gastric folds, 0.78 for the pinch at the distal oesophagus, and 0.85 for the diaphragmatic hiatus. They concluded that the reliability of using the Prague C&M criteria for the endoscopic grading of Barrett's oesophagus is excellent, although endoscopic recognition of lengths of Barrett's less than 1 cm have very low reliability. Nevertheless, they felt that the Prague C&M criteria represent a major advance in the endoscopic recognition and grading of Barrett's oesophagus. The group accepts the descriptive limitations of this system, for example proximal isolated islands of columnar lined oesophagus cannot be specified, and acknowledge that the agreement data presented was in the artificial setting of experts watching a video without performing the endoscopy themselves. Moreover, whilst conscious of the fact that all current endoscopes have only 5 cm incremental markings, the expert panel could continually monitor centimeter distances on screen.

To date, there has been no published validation of the Prague C & M criteria in a live endoscopy setting.

Figure 1.1 Diagrammatic representation of the anatomic landmarks of the oesophago-gastric junction in the setting of Barrett's oesophagus with a hiatus hernia. The classification of the lengths of circumferential (C) and maximal extent (including non-circumferential tongues, M) by the Prague C & M system is indicated.



1.1.2 Arachidonic acid metabolism and Barrett's oesophagus

One of the molecular pathways in carcinogenesis that may be amenable to therapeutic modulation are enzymes that metabolize polyunsaturated fatty acids (PUFA); principally arachidonic acid and its parent compound, linoleic acid (the predominant PUFA in the human diet). Polyunsaturated fatty acids can enhance tumorigenesis in animal models (Phinney 1996). Arachidonic acid is present in virtually all cells and is released from membrane phospholipids by phospholipase A_2, and then metabolized by two major enzyme pathways. Prostaglandins and thromboxane are produced by cyclooxygenase (COX), and leukotrienes and hydroxyeicosatetranoic acids by lipoxygenases (LO). Prostaglandins and leukotrienes are potent inflammatory mediators and have been shown to play a role in mediating the inflammation seen in reflux oesophagitis (Triadafilopoulos et al. 1996).

Cyclooxygenases are integral membrane glycoproteins that function as rate-limiting enzyme in the synthesis of prostaglandins. Two isoforms have been identified. COX-1 is constitutively expressed and plays important physiological roles (Vane 1994). COX-2 is an inducible form, its expression initiated by a number of various stimuli (Kargman et al. 1996). Increased expression of COX-2 has been shown both in animal models of inflammation and in human inflammatory diseases (Pritchard, Jr. et al. 1994;Fu et al. 1996). Recent work has shown that COX-2 expression plays a significant role in progression of Barrett's metaplasia to dysplasia and carcinoma (Shirvani et al. 2000;Bhandari et al. 2001). This is also supported by several epidemiologic studies which have shown that the use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs), by inhibiting COX activity, can reduce the risk of oesophageal and gastric cancers (Thun 1996). Based on this evidence, a large multicentre interventional study (AspECT) is in progress to see whether suppression of COX by aspirin can reduce the progression of Barrett's metaplasia to dysplasia and cancer (Das et al. 2009;2010b).

The role of the cyclooxygenase pathway of arachidonic acid metabolism in carcinogenesis has received much attention, but much less is known about the lipoxygenase pathway. There is evidence that cyclooxygenase and lipoxygenase enzymes share a dynamic relationship and that suppression of COX, can increase eicosanoid production along the LO pathway (Moussard et al. 1988;Schuligoi et al. 1998). With removal of PGE_2, the deleterious effect of LTs could be enhanced by removing the brake on LT synthesis (the so-called "PGE_2 brake" hypothesis) or by shunting their common precursor, arachidonate, down the leukotriene pathway (the "shunting" hypothesis) (Sampson & Holgate 2004;Szczeklik 1990;Szczeklik 1995;Sestini et al. 1996).

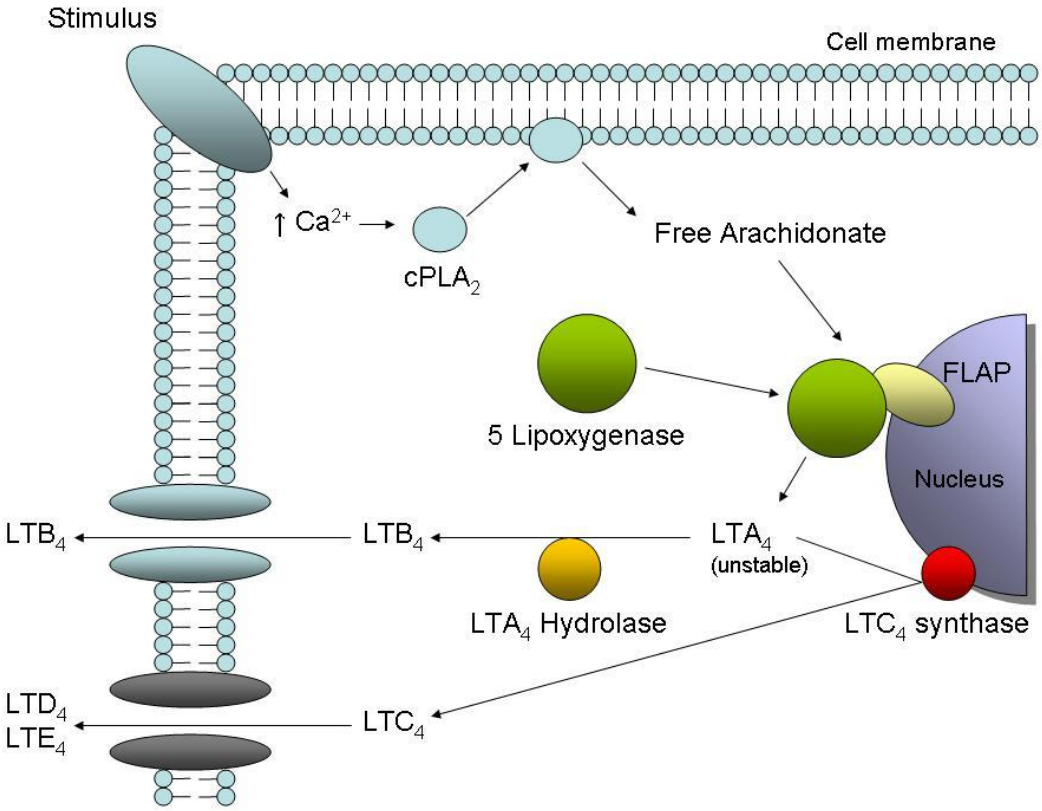
The lipoxygenases comprise a family of non-haem iron-containing dioxygenases that catalyze the oxygenation of arachidonic acid into hydroxyeicosatetranoic acids, leukotrienes (LT), or lipoxins depending on the biosynthetic capacity of each cell type (Needleman et al. 1986). From arachidonic acid the 5-, 8-, 12-, and 15-LO-2 enzymes produce their respective hydroxyeicosatetranoic acids (5-, 8-, 12-, and 15-HETE). The oxidative metabolism of linoleic acid is limited however to the 15-LO-1 pathway, which produces 13-S-HODE (Daret et al. 1989). The precursor for leukotriene synthesis, 5-hydroperoxyeicosatetraenoic acid (HPETE) is produced from arachidonate following the calcium-dependent activation of 5-LO by an 18kD membrane protein 5-LO activating protein (FLAP), the association corresponding with a translocation of 5-LO from a cytoplasmic or intranuclear locations to the nuclear envelope (Figure 1.2) (Dixon et al. 1990;Peters-Golden 1998). 5-HPETE spontaneously degrades to 5-HETE or in the presence of 5-LO it is oxidized to the unstable epoxide leukotriene A_4. LTA_4 can be converted to LTB_4 by the action of cytosolic LTA_4 hydrolase (LTA_4H), or conjugated with the tripeptide glutathione to form the first of the sulphidopeptide- or cysteinyl-leukotrienes by LTC_4 synthase, a dimeric enzyme that, like FLAP, is also embedded within the nuclear membrane (Shimizu et al. 1986;Christmas et al. 2002). LTB_4 and LTC_4 are released from cells by specific export carriers. LTB_4 acts at 7-transmembrane G-protein coupled receptors named BLT1 or BLT2 receptors on target

cells (Yokomizo et al. 1997;Yokomizo et al. 2000), while LTC_4 can be converted by ubiquitous extracellular enzymes to LTD_4 and LTE_4, all of which cysteinyl-leukotrienes act at CysLT receptors, of which three types have been described so far ($CysLT_1$, $CysLT_2$, GPR17) (Lynch et al. 1999b;Beller et al. 2004;Ciana et al. 2006).

The BLT1 receptor mediates most, if not all, of the chemoattractant and proinflammatory actions of LTB_4 (Tager & Luster 2003). The BLT2 receptor has a lower affinity for LTB_4, and binds other LO products, little is known about its role. $CysLT_1$ receptor mediates sustained bronchoconstriction (smooth muscle), mucus secretion and oedema in the airways. The expression of $CysLT_1$ can be affected at the transcriptional level by cytokines known to promote the type 2 helper T cell (Th2) response (Espinosa et al. 2003). Furthermore, leukotrienes can amplify the inflammatory response mediated by these Th2 T cells (Robbiani et al. 2000;Okunishi et al. 2004;Parameswaran et al. 2004). The cytokine milieu in Barrett's oesophagus is different to that of normal squamous-lined oesophagus, and indeed that of oesophagitis (Fitzgerald et al. 2002b). Fitzgerald *et al* demonstrated an inflammatory gradient in the Th2 cytokines interleukin (IL)-4 and IL-10 in Barrett's oesophagus, with maximal inflammation seen at the new squamocolumnar junction (increased pro-inflammatory IL-8 and IL-1β), with an increase in IL-4 and IL-10 distally in the Barrett's segment (where tumours tend to occur)(Fitzgerald et al. 2002b;Fitzgerald et al. 2002a). Expression of LTC_4 synthase is up-regulated by IL-4 (Hsieh et al. 2001). The $CysLT_1$ receptor is the target of the lukast class of leukotriene receptor antagonists used in asthma and allergy therapy, such as montelukast. Other leukotriene modifiers including BLT receptor antagonists and FLAP inhibitors are also under development. The $CysLT_2$ receptor contributes to inflammation, vascular permeability and tissue fibrosis. As a group, leukotrienes promote the chemotaxis and function of the majority of leukocyte subgroups. As mentioned above, any genetic predisposition to Barrett's oesophagus will be polygenic, polymorphisms of the coding and promoter regions of the genes for 5-LO and FLAP have been described (In et al. 1997;Helgadottir et al. 2004).

As with the prostanoids, there has been significant interest in the possible roles of lipoxygenase products, including leukotrienes, in tumorigenesis. While lipoxygenase products have diverse actions, it seems in general that there is a group with pro-inflammatory and procarcinogenic effects (products of 5-, 8-, 12-LO), whilst products of the two iosenzymes of 15-LO possess broadly anti-inflammatory and anticarcinogenic effects (Shureiqi & Lippman 2001). Lipoxygenase expression has been well documented in many solid tumour cells, including prostate, colon, stomach, and oesophagus (Hong et al. 1999;Hoque et al. 2005;Chen et al. 2004). Many LO products have been linked to tumorigenesis in *in vitro* and *in vivo* models, and early studies of LO inhibitors suggested anti-tumorigenic effects (Hussey & Tisdale 1994;Steele et al. 1999).

Figure 1.2 Schematic representation of arachidonic acid metabolism via the 5-Lipoxygenase pathway, with the generation of LTB_4 and the cysteinyl-leukotrienes LTC_4, LTD_4 and LTE_4 ($cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-Lipoxygenase activating protein).



It has been shown that LTD_4 enhances intestinal epithelial cell survival by activating β-catenin signaling, promoting the association of β-catenin with the antiapoptotic protein Bcl-2 (Mezhybovska et al. 2006). In the oesophagus, increasing 5-LO expression through normal, Barrett's, dysplastic mucosa and adenocarcinoma was observed in one series (Chen et al. 2004). In another study, 5-LO was expressed in the majority of both squamous and adenocarcinomas as well as a range of cell lines. 5-LO inhibitors produced an increase in apoptosis, associated with reduced levels of LTB_4 (Hoque et al. 2005). In a rat model of oesophageal adenocarcinoma, increased levels of 5-, 8-, 12-, 15-HETE, LTB_4, and PGE_2 compared to the proximal oesophagus were found by mass spectrometry (Chen et al. 2002). In two studies using the same model, a reduced incidence of adenocarcinoma was observed with both broad (sulindac, NDGA) and selective (celecoxib, zileuton) inhibitors of COX-2 and 5-LO respectively (Chen et al. 2002;Chen et al. 2004). There is also evidence for COX-independent effects of NSAIDs, with the pro-apoptotic 15-LO-1 & 2 found to be down-regulated in oesophageal tumour cells (Shureiqi et al. 2001;Xu et al. 2003). Expression was restored by COX inhibitors, with a subsequent increase in the product 13-S-HODE and a reduction in cell proliferation. This effect was prevented with a specific inhibitor, then restored with exogenous 13-S-HODE (Shureiqi et al. 2001).

The molecular study of the relationship between polyunsaturated fatty acid metabolism and carcinogenesis is revealing novel molecular targets for cancer chemoprevention. COX-2 inhibitors have a proven role in chemoprevention, but their role in Barrett's related adenocarcinoma and their interactions with the lipoxygenase family needs to be established. Preliminary work from our group suggested a significant increase in 5-LO and FLAP expression in oesophageal adenocarcinoma compared to Barrett's oesophagus (unpublished).

1.1.3 Oncogene amplification in Barrett's oesophagus and associated adenocarcinoma

The search for biomarkers to aid clinical management and prognostication in Barrett's and related adenocarcinoma have included the *HER-2/neu (HER-2)* oncogene located on chromosome 17, and its product $p185^{HER-2/neu}$ (HER-2) a transmembrane growth factor receptor of the epidermal growth factor receptor family (al Kasspooles et al. 1993;Walch et al. 2000b;Walch et al. 2000a;Walch et al. 2001). Over-expression of HER-2 protein or amplification of the *HER-2* gene using FDA-approved techniques in invasive breast cancer is associated with poor survival and is used to identify patients who may respond to treatment with the humanized monoclonal antibody trastuzumab (Herceptin®, Roche pharmaceuticals) (Ross & Fletcher 1999). The majority of studies of HER-2 over-expression in tumours of the gastrointestinal tract indicate a potential prognostic value, with the exciting potential for new therapeutic approaches (Ross & McKenna 2001;Tanner et al. 2005). Recently presented work has demonstrated improved overall and progression free survival in a phase III randomized control trial (ToGA trial) in advanced gastric carcinoma that were highly HER-2 positive (IHC2+/FISH+ or IHC3+) with cisplatin and capecitabine/5-fluorouracil alone or in combination with trastuzumab (Van Cutsem et al. 2009). The National Institute for Health and Clinical Excellence (NICE) is about to issue final guidance on the use of trastuzumab in advanced HER-2 positive gastric carcinoma (2010a).

Previous immunohistochemical studies have demonstrated marked variation in the presence of HER-2 over-expression in oesophageal adenocarcinoma (11% to 73%), with no staining generally seen in non-dysplastic Barrett's oesophagus (Jankowski et al. 1992;Flejou et al. 1994;Nakamura et al. 1994;Hardwick et al. 1995;Hardwick et al. 1997;Polkowski et al. 1999). Amplification of *HER-2* gene copy number was assessed by Southern blot analysis or differential PCR in two studies, and found to be present in 15.4% and 14% of oesophageal adenocarcinomas(al Kasspooles et al. 1993;Geddert et al. 2002). Four studies have assessed *HER-2* gene amplification using dual colour

fluorescence *in situ* hybridization (FISH), and found gene amplification to be present in 32% to 83.3% of Barrett's-associated adenocarcinomas (Walch et al. 2004;Walch et al. 2001;Rossi et al. 2010;Walch et al. 2000a). This technique has the potential to allow differentiation of cells with *HER-2* gene amplification from those with chromosome 17 aneusomy. Chromosomal aneusomy is a frequent early event in Barrett's-associated carcinogenesis, occurring in 60-100% of adenocarcinomas (Beuzen et al. 2000;Ferrando et al. 1998). In 3 of 9 cases of gastric carcinoma that displayed co-amplification of *HER-2* and *Topo IIα*, high expression of Topo IIα by Western blotting was present in only one (Kanta et al. 2006). Intense HER-2 expression has been described in oesophageal adenocarcinomas with gene amplification, while moderate and weak protein expression has been identified in tumours with chromosome 17 polysomy alone and in other tumours showing no specific oncogene amplification (Walch et al. 2001;Geddert et al. 2002). This phenomenon has also been observed in cancers of the breast, ovary, salivary gland and endometrium (Jimenez et al. 2000;Underwood et al. 1995;Press et al. 1994;Saffari et al. 1995). Therefore there may be regulation of protein expression at the transcriptional or post-transcriptional level, as well as due to oncogene amplification (Geddert et al. 2002).

The prognostic significance of HER-2 over-expression and *HER-2* gene amplification in oesophageal adenocarcinoma remains uncertain (Ross & McKenna 2001). Brien and co-workers reported the presence of *HER-2* gene amplification in 19% (n=63) of cases using a locus-specific probe only; in this study, the presence of multiple *HER-2* gene copies was an independent predictor of poor outcome (Brien et al. 2000). However, other studies have found increased *HER-2* mRNA levels and intense protein expression only in tumours with gene amplification compared to those with chromosome 17 polysomy but no amplification (Walch et al. 2001).

In a previous study by our group, we investigated HER-2 protein over-expression and gene amplification in archival tissue from 100 resected oesophageal adenocarcinomas using immunohistochemistry (Dako Herceptest®) and fluorescence *in situ*

hybridization (FISH, Vysis PathVysion®) respectively (Dr B Stacey, Southampton University Hospitals, Southampton, UK, submitted for DM). A minimum of five years clinical follow up was available. The relationship of protein over-expression and gene amplification to histopathological tumour characteristics and survival was determined.

HER-2 gene amplification was identified in 12 cases (12%), with HER-2 over expression (scoring 2+ or 3+ using the Herceptest IHC protocol) in four of these cases ($p<0.01$). *HER-2* gene amplification and chromosome 17 polysomy co-existed in two cases. Chromosome 17 polysomy alone was present in 26 cases (26%), with two of these over-expressing HER-2 ($p=0.85$). No HER-2 over-expression was seen in the 62% of tumours that were chromosome 17 diploid and without *HER-2* gene amplification. There was a trend to a worse survival on Kaplan-Meier analysis with both chromosome 17 polyploidy ($p=0.09$) and *HER-2* amplification ($p=0.1$). The survival of these groups when combined was significantly worse ($p=0.03$) than tumours that were diploid for chromosome 17. Cox regression analysis indicated that tumours with *HER-2* gene amplification or chromosome 17 polyploidy (HR=1.86, 95% CI: 1.18 – 2.93, $p<0.01$) had higher risk of death than patients with disomy. There was no association between *HER-2* gene amplification or chromosome 17 polysomy and histopathological tumour characteristics (Stacey et al. 2006).

We concluded that HER-2 over-expression and *HER-2* gene amplification is identifiable in some oesophageal adenocarcinomas (HER-2 'positive') using FDA-approved methods for predicting trastuzumab response in breast cancer. It is possible that these HER-2 'positive' poor prognosis tumours may also respond to trastuzumab therapy. However, a significantly higher risk of death was only seen when cases with *HER-2* gene amplification or chromosome 17 polyploidy were combined, suggesting the presence of other oncogenic mechanisms underlying the more aggressive behaviour of these two subgroups.

Located close to *HER-2* on chromosome 17 is the gene that encodes the protein topoisomerase II alpha (Topo IIα). Topo IIα is a key enzyme in DNA replication, excising and reconnecting double-stranded super-coiled DNA during the replicative cell cycle, and a target of many chemotherapeutic agents (Froelich-Ammon & Osheroff 1995). Topo IIα belongs to a family of topoisomerases that routinely break and join DNA chains, altering the topological state of the DNA (Wang 1996;Champoux JJ 2001). They accomplish this action by either passing one strand of DNA through a break in the opposite strand (type I subfamily) or by passing a section of double stranded DNA through a double-stranded gap in the DNA (type II subfamily). As well as altering the supercoiling of a closed DNA domain, topoisomerases can unravel entwined linear chromosomes (Giaever et al. 1988). Once unraveled, transcription or replication can begin, with the topoisomerase transforming the relaxed DNA back into a condensed chromosome when completed.

As described, topoisomerase II cleaves both strands of the DNA, a "gate DNA" (G-DNA) in an ATP-dependent process, allowing the transport of a duplex of DNA (T-DNA) through the gap, and then joins the cleaved DNA (Berger 1998). In eukaryotes, the enzyme is a homodimer, with two protein chains each containing an A and B subunit. The B subunit contains the ATPase activity, and is N-terminal to the A subunit that contains the DNA-cleaving active site. In between these regions lies the B' domain, responsible for DNA binding and structural interactions. The C terminal portion is the least homologous between species, and is involved with transferring the T-DNA. Topoisomerase II enzymes are involved in many genetic processes, including the separation of chromosomes in prokaryotic and eukaryotic cells (Wang 2002;Porter & Farr 2004). Initially all type II enzymes were thought to belong to the same super family, but the discovery of a novel isoform led to the formation of A and B subfamilies. Within the A subfamily are two isoforms with different molecular weights, cleavage sites and catalytic capacity termed topoisomerase IIα and IIβ (Drake et al. 1987;Drake et al. 1989). Topoisomerase IIα is preferentially expressed in proliferating cells in the cell cycle, topoisomerase IIβ is expressed in equal amounts in proliferating

and non-proliferating cells and is located in the nucleolus, suggesting a role in DNA transcription (Heck & Earnshaw 1986;Heck et al. 1987;Woessner et al. 1991). Topoisomerase II protein levels vary through the cell cycle, peaking in late S and G2/M phases (>10 times that of G1) (Woessner et al. 1991;Goswami et al. 1996). This corresponds with their role in the untangling, condensing and mitotic separation of daughter chromatids (Porter & Farr 2004;Schvartzman 2004;Espeli & Marians 2004;Cortes et al. 2003).

With understanding of the crucial role played by topoisomerase II in DNA topology came the search for drugs to target this key family of enzymes, resulting in a number of anti-microbial and anti-cancer chemotherapeutic agents. Anthracyclines are potent anti-cancer agents that intercalate with the G-DNA, covalently linking it to topoisomerase II, and preventing the DNA from rejoining and inducing apoptosis (Minotti et al. 2004;Binaschi et al. 2001).

The gene encoding Topo IIα, *topoisomerase II alpha* (*Topo IIα*), is located at chromosome band 17q12-q21, close to *HER-2* on chromosome 17 (Smith et al. 1993). In breast cancer, *HER-2* is a commonly amplified oncogene and many cases also show *Topo IIα* gene amplification or deletion: 41-44% and 42% respectively (Jarvinen et al. 2000;Jarvinen et al. 1999). Amplification of *Topo IIα* with corresponding over-expression of the protein product provides a greater "target" for anthracycline-based chemotherapy, with a predicted increased response (Coon et al. 2002;Jarvinen et al. 2000). Conversely, *Topo IIα* deletion may result in a resistance to anthracycline-based therapy.

The expression of *Topo IIα* in oesophageal tumours has only been studied in two papers to date. Recently, Rossi *et al* have published their study of Topo IIα and HER-2/neu over-expression/amplification in Barrett's oesophagus, dysplasia and adenocarcinoma (Rossi et al. 2010). They utilized immunohistochemistry and FISH (Vysis) in 18 cases of Barrett's, 13 cases of Barrett's with dysplasia (5 low grade

dysplasia, 8 high grade dysplasia), and 13 cases of oesophageal adenocarcinoma. In the adenocarcinoma cases, HER-2/*HER-2* was over-expressed/amplified in 61% and 38%, with Topo IIα/*Topo IIα* over-expression/amplification in 69% and 38% respectively. There were no gene deletions in this group of 13 patients. They found a significant association between Topo IIα over-expression and *Topo IIα* amplification, chromosome 17 aneuploidy, *HER-2* amplification, and HER-2 over-expression as well as between HER-2 over-expression and *HER-2* amplification, *Topo IIα* amplification, and chromosome 17 aneuploidy. Gene amplification (*HER-2* or *Topo IIα*), protein over-expression (HER-2 or Topo IIα), and chromosome 17 aneuploidy were associated with dysplasia or adenocarcinoma.

Kim *et al* analysed mRNA expression of oesophageal tumour and adjacent normal tissue by Northern blot analysis in six surgical resection specimens, with a significant difference in mRNA expression of *Topo IIα*. The mRNA expression was correlated with the S-phase population in cell cycle (Kim et al. 1919). No comment is made on the histological type (squamous or adenocarcinoma) of the oesophageal tumours. In the same study, six gastric cancer and six colon cancer resection specimens were analysed, with significantly increased *Topo IIα* mRNA in the tumours compared to adjacent normal tissue. Two other studies have assessed *Topo IIα* amplification in gastric carcinoma. Ming Liu *et al* studied 30 cases of metastatic gastric cancer by FISH and found *HER-2* amplification in 5 (17%), with co-amplification of *Topo IIα* in 2 of these (40%). There was a significant positive correlation between *HER-2* amplification and both *Topo IIα* amplification and Topo IIα over-expression (Ming Liu et al. 2004).

In a study employing chromogenic *in situ* hybridization (CISH) of 131 gastric and 100 gastro-oesophageal junction adenocarcinomas, amplification of *HER-2* was present in 16 (12%) and 24 (24%), with co-amplification of *Topo IIα* in 10 (63%) and 16 (68%) respectively (Tanner et al. 2005). None of the gastric adenocarcinomas without *HER-2* amplification showed *Topo IIα* amplification, the gastro-oesophageal junction

adenocarcinomas without *HER-2* amplification were not studied for *Topo IIα* amplification.

No study has yet explored the potential prognostic significance of Topo IIα over-expression or *Topo IIα* amplification in oesophageal adenocarcinoma.

1.2 Hypothesis and aims

The preceding introduction emphasised the importance of the endoscopic recognition and classification of Barrett's oesophagus, and explored two important cellular processes that may be disturbed in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence.

The aim of my thesis will be to investigate the following hypotheses:

1.2.1 Hypothesis 1: Validation of the Prague C&M classification system for Barrett's oesophagus

We hypothesise:

1. That there will be clinically acceptable agreement between measurements of Barrett's oesophagus in a clinical setting on push and pull endoscopy with a single endoscopist.
2. That there will be clinically acceptable agreement between measurements of Barrett's oesophagus in a clinical setting on pull endoscopy between two endoscopists.
3. The presence of a hiatus hernia should not reduce agreement between measurements of Barrett's oesophagus in a clinical setting on pull endoscopy between two endoscopists.

1.2.2 Hypothesis 2: 5-Lipoxygenase over expression occurs in Barrett's metaplasia, dysplasia, and adenocarcinoma

We hypothesise secondly:

1. That 5-LO, FLAP and COX-2 protein expression will correlate with progression along the metaplasia-dysplasia-adenocarcinoma sequence.
2. That the capacity of oesophageal biopsies to generate leukotrienes B_4 and C_4 *in vitro,* either spontaneously or in response to a calcium ionophore (calcimycin A23187), will correlate with progression along the metaplasia-dysplasia-adenocarcinoma sequence.

1.2.3 3: Topoisomerase II alpha over-expression and gene amplification in oesophageal adenocarcinoma.

We hypothesise thirdly:

1. That *topoisomerase II alpha* amplification or deletion is a frequent genetic event in oesophageal adenocarcinoma.
2. That amplification/deletion correlates with protein expression, *HER-2* amplification, histopathological tumour characteristics and clinical outcome.

2 GENERAL METHODS

The methods used repeatedly throughout the course of this thesis are described in this section, including any methodological development where appropriate. The materials used are listed in the Appendix.

2.1 Immunohistochemistry

Three micron thick sections were cut from formalin-fixed and paraffin-embedded tissue blocks and mounted on adhesive positively-charged Superfrost Plus slides (Menzel-Glaser, Germany), and dried overnight at 37°C. Sections were deparaffinised using xylene, re-hydrated through alcohol and treated in 0.5% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxide activity, then washed in water. A high temperature antigen unmasking technique was then carried out in 0.01 M citrate buffer (pH 6.0) for 6 minutes in a pressure cooker. Slides were washed in water, then twice with Tris buffered saline (TBS, pH 7.6), and then again with Menapath Wash (Menarini Diagnostics Ltd, UK) to enhance removal of wash prior to application of antibody solution. Slides were incubated with 200µl of appropriately-diluted primary antibody for 30 minutes at room temperature. After washing with TBS then Menapath Wash, a standard avidin-biotin-peroxidase complex technique using the Vectastain Universal Elite kit (Vector Labs Inc., USA) was applied for visualization. Biotinylated horse anti-rabbit/mouse immunoglobulin G and avidin label were each applied for 30 minutes at room temperature, before washing in TBS and Menapath Wash. Finally the slides were treated with 200µl of the substrate-chromagen solution DAB (5% 3,3'-diaminobenzidine tetrahydrochloride) for 10 minutes at room temperature. After a wash in running water for 15 minutes, Mayer's haematoxylin counter stain was applied for 2 minutes prior to a further wash in water and differentiation in 1% acid alcohol, and left to blue in running water. The slides were then dehydrated in 70% and absolute alcohol baths, cleared in xylene and mounted.

2.1.1 Immunohistochemistry for topoisomerase IIα

An initial antibody dilution was performed on sections of human tonsil (surplus to diagnostic requirements), containing proliferating lymphoid tissue. Sections for a negative control and dilutions of 1:20, 1:40, 1:80, and 1:160 of mouse monoclonal anti-human Topo IIα antibody (Novocastra, Newcastle, UK) were processed as in section 2.2. The optimum staining was given with the 1:40 dilution.

2.1.2 Immunohistochemistry for components of the 5-LO pathway

An initial antibody dilution was performed on sections of human tonsil (surplus to diagnostic requirements), containing proliferating lymphoid tissue. Sections for a negative control and dilutions of 1:20, 1:40, 1:80, and 1:160 of mouse anti-human polyclonal antibodies directed against 5-lipoxygenase, FLAP (kindly donated by Dr J Evans, Merck & Co, West Point, PA) and mouse anti-human monoclonal antibodies against COX-2 (Cayman Chemical Inc, Ann Arbor, MI, USA) were processed as in section 2.2. The optimum staining was given with the 1:40 dilution.

2.1.3 Fluorescence *in situ* hybridization

An initial control slide of isolated peripheral blood leukocytes was kindly prepared by Adam Stewart (Cytogenetics Group, Cancer Sciences Division, University of Southampton, UK). Thereafter, 3 micron sections from formalin-fixed and paraffin-embedded tissue blocks, consecutive to those used for immunohistochemistry for topo IIα in the case of oesophageal adenocarcinoma blocks, were cut and mounted on adhesive positively charged Superfrost Plus slides (Menzel-Glaser, Germany), and dried overnight at 37°C. Sections were deparaffinised using xylene, re-hydrated through alcohol then air dried for 5 minutes. A paraffin pretreatment reagent kit (Abbott Molecular Inc, IL, USA) was then used on all paraffin sections according to the manufacturer's instructions. The slides were pre-treated with 0.2 M HCl for 20

minutes, an acid permeabilisation step to reduce the subsequent proteolytic step to minimize tissue damage. The slides were then washed in purified water, and then 2 x SSC (pH 7.0) before being immersed in sodium thiocyanate (pretreatment solution) at 80°C for 30 minutes, followed by two washes in 2 x SSC. Protease treatment at 37°C for 22 minutes followed, using 62.5-75 MU lyophilized protease in 50ml of NaCl (pH 2). This step removes cellular cytoplasm, allowing the probe to penetrate the nucleus. Slides were then washed twice in 2 x SSC and dried on a slide warmer at 45°C. Dry slides were fixed with 10% buffered formalin (4% formaldehyde, Buffered Formaldehyde Concentrate, Genta medical, UK), washed with 2 x SSC and then dehydrated with graded ethanols (70%, 85%, 100%). Seven microlitres of directly labeled fluorescent DNA probes specific for the *Topo IIα* gene locus and the alpha satellite DNA sequence at the centromeric region of chromosome 17 were applied to the slides (Locus Specific Identifier® TOPO2A/CEP17 probe mixture, Abbott Molecular Inc, IL, USA). A cover slip was then sealed in place with rubber cement. The sections were denatured at 75°C for 5 minutes and hybridization took place overnight at 37°C (HYBrite co-denaturation, Abbott Molecular Inc, IL, USA). Post-hybridization washing was carried out with 2 x SSC at room temperature (2 minutes), 0.4 x SSC with 0.3% NP-40 at 73 °C (2 minutes) and 2 x SSC with 0.1% NP-40 at room temperature (2 minutes). Counterstaining of nuclei was performed using 10µl of 4,6-diamino-2-phenylinolide (DAPI, Abbott Molecular), then cover slipped.

2.1.4 Fluorescence *in situ* hybridization interpretation

The slides were evaluated using a triple filter fluorescence light microscope with x100 oil immersion objective with the aid of MacProbe v4.3.2 software. A DAPI single-band pass filter was used to visualise nuclei, a green single-band pass filter to visualise spectrum green CEP-17 centromeric marker, and an orange single-band pass filter to visualise the spectrum orange TOPO2A *Topo IIα* gene locus. The area of tumour was identified and signals from at least 20 non-overlapping tumour nuclei were counted to

determine the mean number of signals per cell for each probe. Polymorphonuclear cells were used as positive controls.

A tumour was considered to show *Topo IIα* gene amplification if the ratio of *Topo IIα* gene signals to centromeric chromosome 17 signals was greater than 2. Deletion of the *Topo IIα* gene was regarded as anything below a ratio of 0.8.

2.2 Quantification of LTB_4 and Cys-LT production of whole biopsy specimens by enzyme immunoassay

Standard biopsies from the foregut were taken at endoscopy using standard biopsy forceps. The wet weight of the biopsy was obtained. The biopsy was placed into Dulbecco's Modified Eagle's Medium (DMEM, Sigma, Poole, UK) with 20 mM 4-(2-hydoroxyethyl)-1-piperazin ethanesulfonic acid (HEPES, Sigma) and allowed to equilibrate for 5 min at 37°C. A stock solution of calcium ionophore, Calcimycin A23187 free acid (Sigma, Poole, UK) 2 mM in 100% dimethyl sulfoxide (DMSO, Sigma) was prepared. A working stock was prepared by diluting 10-fold in PBS to give 200 uM A23187 (in 10% DMSO). The calcium ionophore solution was added to the biopsy in DMEM, with a further 100 fold dilution, to give a final A23187 concentration of 2 uM in contact with the biopsy and a DMSO concentration not exceeding 0.1% v/v (e.g. 10 ul into 1000 ul). This was incubated for a range of time periods. The reaction was stopped with 2-4 volumes of ice-cold methanol (to denature protein and hence terminate 5-LO pathway enzyme activity). The sample was then left in the methanolic solution overnight at 4°C to extract intracellular leukotrienes from cells and to reduce binding to the extracellular matrix. To pellet the biopsy and precipitate free protein, the sample was centrifuged at 2500 RPM for 5 minutes (RCF: 776g). The lipid-containing supernatant was decanted into a clean tube and frozen at -20°C. The methanol was evaporated to dryness using a Gyrovap rotary evaporator (Howe & Co, London, UK), which centrifuges up to 40 samples at one time to prevent bubbling over when under reduced atmospheric pressure, allowing rapid evaporation at room temperatures. A condenser was used to trap the evaporated methanol to waste.

For enzyme immunoassay, dried samples were resuspended in 250 ul EIA buffer. Two 50 ul aliquots each for LTB_4 and total cysteinyl-leukotrienes ($LTC_4/D_4/E_4$) were quantified using Cayman Chemicals EIA kits as per the manufacturer's instructions, against authentic leukotriene standards over the range (LTB_4: 500 to 3.9 pg/ml, cys-

LT: 1000 to 7.8 pg/ml). The plates were analysed with a Spectramax 340pc and Softmax Pro software (Molecular Devices, Sunnyvale, CA) at 405 nM. The LTB_4 primary antibody has a detection limit of 13 pg/ml, and cross-reactivity specificity of: LTB_4 100%; 5(S)-HETE 6.6%; 5(R)-HETE 3.7%; 20-hydroxy LTB_4 2.7%; others <1%, with cys-LT <0.001%). The cys-LT primary antibody has a detection limit of 34 pg/ml, and cross-reactivity specificity of: LTC_4 100%; LTD_4 100%; LTE_4 67%; LTD_5 61%; LTC_5 54%; LTE_5 41%; N-acetyl LTE_4 10.5%; others <0.01%. The mean amount of LT in the duplicate was corrected to pg per mg wet weight of biopsy.

2.2.1 Calcimycin A23187 serial dilution preparation

Ten ul of the 2 mM stock solution (100% DMSO) were added directly to the 1 ml of DMEM, to give 20 uM (1% DMSO). Ten ul of the 200 uM working stock (10% DMSO) were added to the 1 ml of DMEM to give 2 uM (0.1% DMSO). An intermediate stock was made by adding 10 ul of working stock to 90 ul of PBS, and 10 ul of this was added to the 1 ml of DMEM to give 0.2 uM (0.01% DMSO).

2.2.2 FLAP inhibitor MK866 preparation

In some experiments, the FLAP inhibitor MK886 (Cayman Chemicals Inc) was used to block production of leukotrienes in biopsy cultures in order to confirm that LTB_4 and cys-LTs detected by immunoassay are authentic 5-LO/FLAP pathway products (Dixon et al. 1990). Stock solutions of MK-886 were made up in DMSO and the drug was applied to biopsies at a final concentration of 10 uM at 10 min before the addition of A23872.

3 INTRA- & INTER-OBSERVER AGREEMENT IN BARRETT'S OESOPHAGUS USING THE PRAGUE C & M CRITERIA: A LIVE ENDOSCOPY EVALUATION

3.1 Introduction

The first step in the diagnosis of Barrett's oesophagus is the endoscopic identification of any portion of the normal squamous lining that has been replaced by a metaplastic columnar epithelium (Sampliner & Practice Parameters Committee of the American College of Gastroenterology 2002;Playford 2006). Histological assessment of biopsy specimens confirms the presence of intestinal metaplasia and the diagnosis of Barrett's oesophagus, the premalignant lesion for oesophageal adenocarcinoma (Naef et al. 1975;Haggitt et al. 1978). The incidence of oesophageal adenocarcinoma has increased at a greater rate than any other solid tumour in the Western world in recent years (Blot 1994), with malignant risk increasing with increasing length of Barrett's oesophagus (Iftikhar et al. 1992;Menke-Pluymers et al. 1993;Gopal et al. 2003). Furthermore, there is conflicting evidence that acid suppression with proton pump inhibitors (PPI) may decrease the length of Barrett's oesophagus. Results from prospective studies of 13-68 patients with 2-3 endoscopists demonstrate no significant change in Barrett's oesophagus length, to a mean reduction in Barrett's oesophagus length of 2.6cm (Gore et al. 1993;Malesci et al. 1996;Sharma et al. 1997;Peters et al. 1999;Wilkinson et al. 1999). The largest study of 188 patients found no significant change in Barrett's oesophagus length, but this study was retrospective and involved many endoscopists (Cooper et al. 2006).

A standardized reporting process for Barrett's oesophagus would overcome some of the difficulties comparing different studies of Barrett's oesophagus and allow improved case comparison and recruitment into trials. A first step towards addressing this issue was taken by the development of the consensus-based Prague C & M criteria for the endoscopic reporting of Barrett's oesophagus with standard endoscopic equipment, by a subgroup of the International Working Group on the Classification of Oesophagitis (Sharma et al. 2006). They define the extent of Barrett's oesophagus (the endoscopic appearances suggestive of columnar-lined oesophagus) as the distance between the gastro-oesophageal junction (GOJ) and the squamocolumnar junction, when measured

at the mouth guard, subdivided into the lengths of the Circumferential and Maximum extent of the columnar lined oesophagus. The GOJ was defined as the proximal limit of the longitudinal gastric folds (Sharma et al. 2004;Delvaux & Korman 2000). The validation data presented consisted of the inter-observer agreement between 29 independent assessors who watched 29 endoscopic videos of cases of Barrett's oesophagus on slow pull back of the endoscope after gastric deflation, with a superimposed distance marker displayed in the top left hand corner of the screen. After an introduction to the new classification, assessors were asked to use the C & M criteria on the 29 cases they witnessed. The reliability coefficient for C was 0.94 and M was 0.93. The reliability coefficients for anatomical landmarks were very high, 0.88 for the proximal margin of gastric folds. They concluded that the reliability of using the Prague C & M criteria for the endoscopic grading of Barrett's oesophagus was impressive, although accepting the limitations of the artificial setting.

In this chapter I aimed to explore whether the criteria developed in an artificial setting are valid in a real-life clinical setting, and present the first live endoscopy assessment of intra-observer agreement of the C & M criteria to characterize Barrett's oesophagus with push compared to pull endoscopy by a single endoscopist, and compare the inter-observer agreement with a second endoscopist on pull endoscopy.

3.2 Methods

Forty consecutive patients with Barrett's oesophagus proven by biopsy attending for routine surveillance endoscopy at the Royal South Hants and Southampton General Hospitals were included in this study. All endoscopies were performed using Olympus endoscopes and processors (Olympus Corp, Tokyo, Japan). An experienced endoscopist (Dr D Chan) applied the Prague C & M criteria to record the circumferential and maximal extent of Barrett's oesophagus on push and pull endoscopy (after gastric deflation). The distance from the mouth guard to the proximal margin of gastric folds, the pinch at the distal oesophagus, and the diaphragmatic hiatus were also recorded. A second experienced endoscopist (Dr P Patel), blinded to the initial assessment, repeated the set of measurements on pull endoscopy (with the first endoscopist not present). Landmark measurements and calculation of the C & M values were recorded by an independent observer (myself), in a manner identical to that used in the Prague criteria development study. Both endoscopists had read the paper detailing the Prague C & M system and were familiar with its application. This study was reviewed by the Southampton and South West Hampshire research ethics committee and designated a service evaluation.

3.2.1 Statistical Analysis

Intra- and inter-rater agreement regarding the length of C & M (a discrete numerical variable) was evaluated by the Pearson correlation coefficient (*r*) and the intraclass correlation coefficient (or reliability coefficient). The intraclass correlation coefficient is equivalent to the weighted *kappa* statistic with quadratic weights, as demonstrated by Fleiss & Cohen and acknowledged by Sharma *et al* (Fleiss & Cohen 1973;Sharma et al. 2006). Therefore the validation study categorized the strength of the reliability coefficient rater agreement according to definitions proposed by Landis and Koch for kappa values (originally designed for categorical data). These were: 0-0.2, slight; 0.21-0.4, fair; 0.41-0.6, moderate; 0.61-0.8, substantial; 0.81-1, almost perfect

(Landis & Koch 1977). To allow direct comparisons to be drawn, we also used these definitions. Furthermore, the proportion of pair wise comparisons in which the endoscopist's measurements agreed exactly, differed by 1 cm, 2 cm or greater was also similarly calculated.

Further assessment of the agreement between observers was made by the approach suggested by Bland and Altman (Bland & Altman 1986). This technique assesses the degree of agreement using the mean difference between measurements, and the standard deviation of the differences. If the differences are Normally distributed, 95% of differences will lie within 2 standard deviations above and below the mean, termed "limits of agreement" by the authors. Provided the differences within these limits of agreement are not clinically important, measurements from the two endoscopists would be equivalent. There is no sample size calculation applicable to this statistical analysis; therefore a reasonable sample group of 40 patients was deemed appropriate for this study.

3.3 Results

Complete data for C & M values and the proximal margin of the gastric folds was collected in all 40 cases of Barrett's oesophagus. The distribution of mean (inter-observer) C & M measurements for the 40 cases is shown in Table 3.1. Simple plots of C & M results for pull versus push endoscopy and first versus second endoscopist are displayed in Figure 3.1-4. The reliability coefficients (Pearson correlation coefficients, r) for pull versus push endoscopy were 0.88 (0.89) for C and 0.87 (0.87) for M ($p<0.001$), and for inter-observer measurements were 0.75 (0.75) for C and 0.76 (0.77) for M ($p<0.001$). These represent "substantial" to "almost perfect" level of reliability (Table 3.2). Comparing pull with push endoscopy, the exact rates of agreement were 38% and 30% for C & M respectively, differing by 1 cm in 75% and 68%, and by 2 cm in 88% and 90% respectively. There were 5 and 4 cases with differences of 3 or 4 cm for C & M respectively. Comparing inter-observer measurements, the exact rates of agreement were 18% and 15% for C & M respectively, differing by 1 cm in 50% and 53%, and by 2 cm in 75% and 73% respectively. There were 9 and 11 cases with differences of 3 or 4 cm for C & M respectively, and 1 case where C differed by 5 cm (Table 3.3).

The absolute intra- and inter-observer differences between the C & M measurements were Normally distributed, shown in Figure 3.5-8. From the Bland-Altman analysis, the mean difference in C between pull and push endoscopy was 0.33 cm (95% confidence interval -0.14 to 0.79), with limits of agreement from -2.59 to 3.24 cm. For M, the mean difference between pull and push endoscopy was 0.63 cm (95% confidence interval 0.15 to 1.10), with limits of agreement from -2.33 to 3.58 cm. The mean difference in C between endoscopists was -0.01 cm (95% confidence interval -0.70 to 0.67), with limits of agreement from -4.30 to 4.27 cm. For M the mean difference between endoscopists was -0.33 cm (95% confidence interval -0.98 to 0.33), with limits of agreement from -4.41 to 3.76 cm (Table 3.4). Bland-Altman plots are shown in Figure 3.9-12.

A sub-group analysis was performed by the presence or absence of a hiatus hernia, and corresponding results presented in Tables 3.5-8. No clinically significant difference in agreement was seen when a hiatus hernia was present or not.

Table 3.1 Number of cases with C & M measurements in relation to the length of the Barrett's oesophagus segment (inter-observer mean).

Estimated BE length (inter-observer mean) (cm)	Number of cases (C value)	Number of cases (M value)
0 to <1	1	0
1 to <3	18	6
3 to <5	6	10
5 to <10	14	21
≥10	1	3

Table 3.2 Reliability coefficients (intraclass correlation coefficients) for recognizing different lengths of Barrett's oesophagus and the position of the proximal margin of the gastric fold for pull compared to push endoscopy, and between endoscopists (inter-observer).

	Reliability coefficient, pull versus push (95% confidence interval)	Reliability (Landis and Koch 1977)	Reliability coefficient, inter-observer (95% confidence interval)	Reliability (Landis and Koch 1977)
Proximal margin of gastric fold	0.85 (0.73-0.92)	Almost perfect	0.78 (0.62-0.88)	Substantial
C value	0.88 (0.78-0.93)	Almost perfect	0.75 (0.57-0.86)	Substantial
M value	0.87 (0.77-0.93)	Almost perfect	0.76 (0.60-0.87)	Substantial

Table 3.3 Agreement by case and percentage for C & M values for push versus pull endoscopy and between endoscopists (inter-observer).

	Agreement, push versus pull, case number (cumulative %)	Agreement, inter-observer, case number (cumulative %)
Exact agreement: C	15 (38)	7 (18)
1 cm difference: C	30 (75)	20 (50)
2 cm difference: C	35 (87)	30 (75)
3-4 cm difference: C	40 (100)	39 (98)
Exact agreement: M	12 (30)	6 (15)
1 cm difference: M	27 (68)	21 (53)
2 cm difference: M	36 (90)	29 (73)
3-4 cm difference: M	40 (100)	40 (100)

Table 3.4 Mean difference with limits of agreement (Bland-Altman analysis), between lengths of Barrett's oesophagus and the position of the proximal margin of the gastric fold for pull compared to push endoscopy, and between endoscopists (inter-observer). CI; confidence interval, SD; standard deviation.

	Mean difference, push against pull (95% CI) (cm)	Lower limit of agreement, mean-2SD (95% CI)	Upper limit of agreement, mean+2SD (95% CI)	Mean difference, inter-observer (95% CI) (cm)	Lower limit of agreement, mean-2SD (95% CI)	Upper limit of agreement, mean+2SD (95% CI)
Proximal margin of gastric fold	0.6 (0.12 to 1.08)	-2.40 (-3.23 to -1.57)	3.60 (2.77 to 4.43)	-0.48 (-1.06 to 0.11)	-4.13 (-5.14 to -3.12)	3.18 (2.17 to 4.19)
C value	0.33 (-0.14 to 0.79)	-2.59 (-3.39 to -1.78)	3.24 (2.43 to 4.04)	-0.01 (-0.70 to 0.67)	-4.30 (-5.49 to -3.11)	4.27 (3.09 to 5.46)
M value	0.63 (0.15 to 1.10)	-2.33 (-3.15 to -1.51)	3.58 (2.76 to 4.40)	-0.33 (-0.98 to 0.33)	-4.41 (-5.54 to -3.27)	3.76 (2.63 to 4.89)

Figure 3.1 Circumferential extent (C) in 40 cases of Barrett's oesophagus using the Prague criteria for pull versus push endoscopy. The line of equality and the Pearson correlation coefficient (*r*) are indicated.

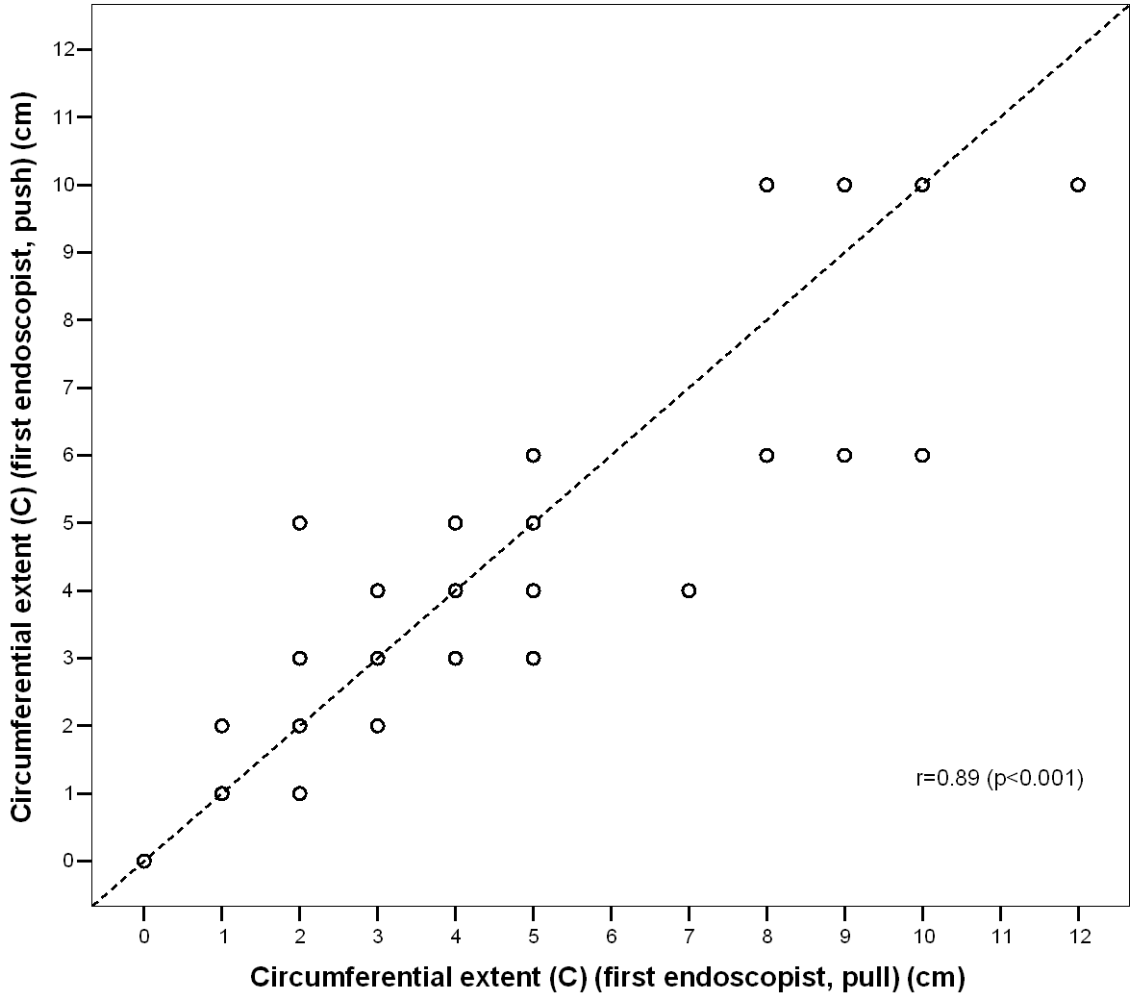


Figure 3.2 Maximal extent (M) in 40 cases of Barrett's oesophagus using the Prague criteria for pull versus push endoscopy. The line of equality and the Pearson correlation coefficient (*r*) are indicated.

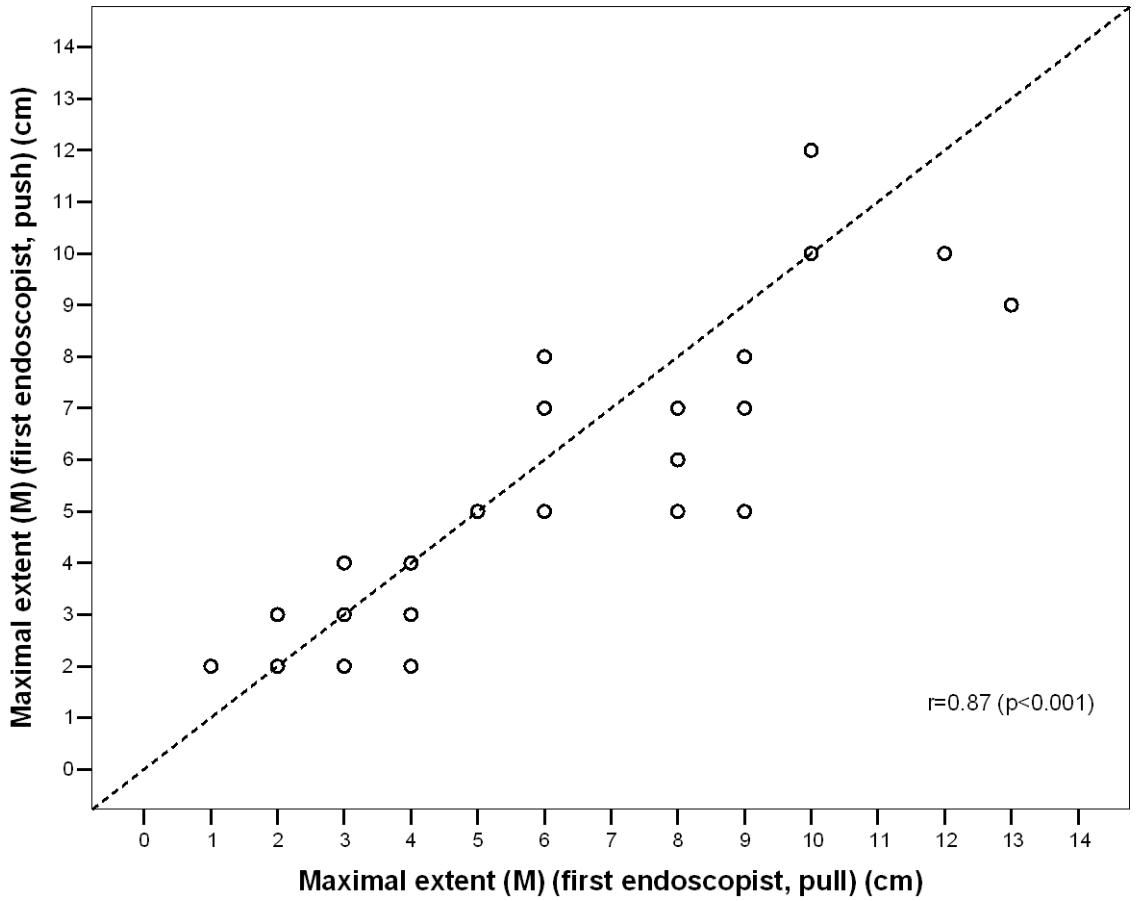


Figure 3.3 Circumferential extent (C) in 40 cases of Barrett's oesophagus using the Prague criteria for first versus second endoscopist on pull endoscopy. The line of equality and the Pearson correlation coefficient (*r*) are indicated.

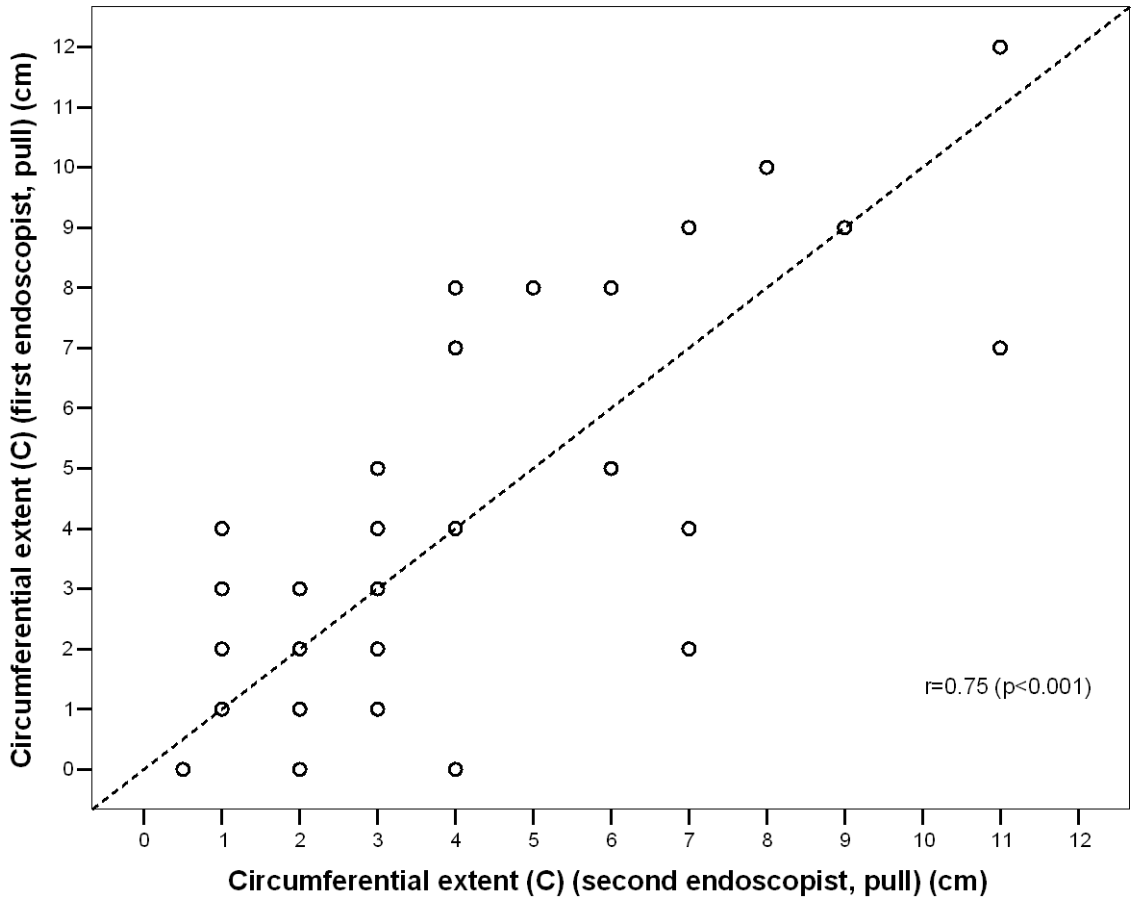


Figure 3.4 Maximal extent (M) in 40 cases of Barrett's oesophagus using the Prague criteria for first versus second endoscopist on pull endoscopy. The line of equality and the Pearson correlation coefficient (*r*) are indicated.

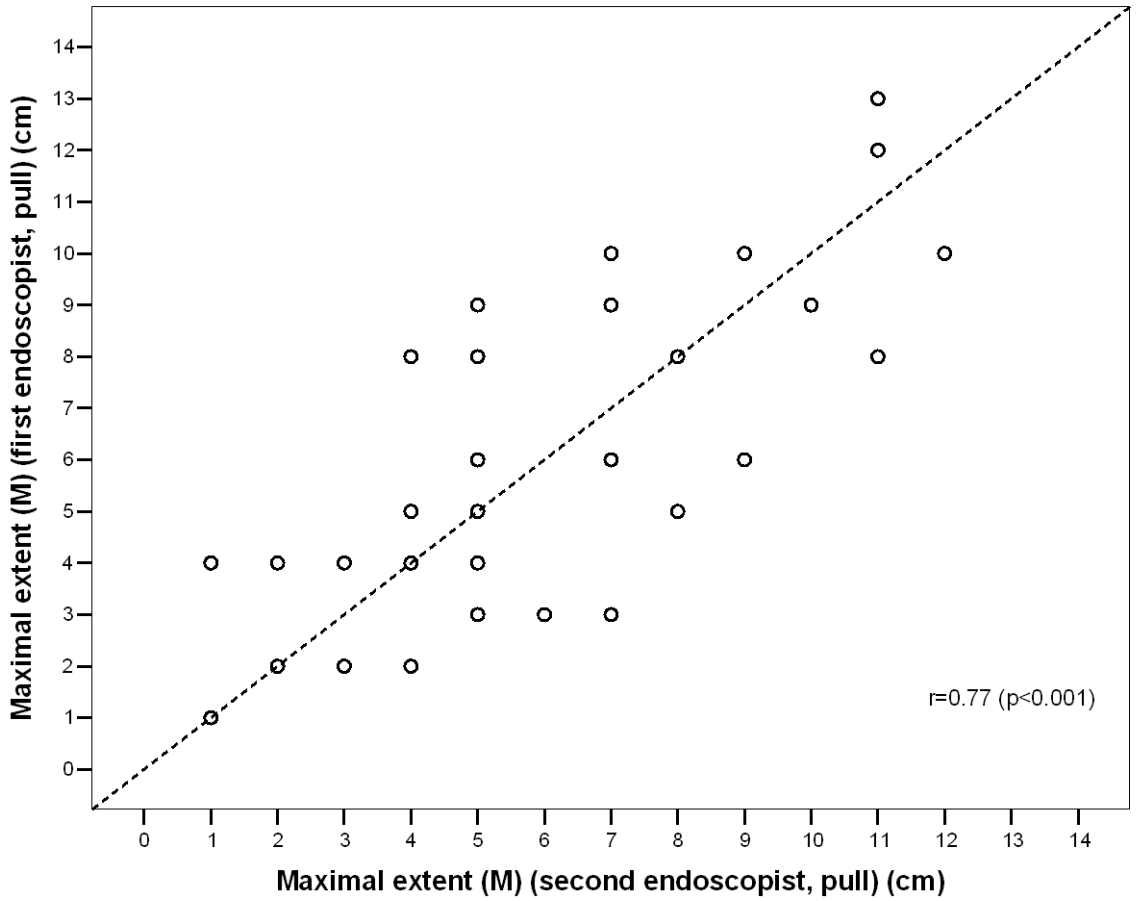


Figure 3.5 Distribution of the differences in C value measurements in 40 cases of Barrett's oesophagus using the Prague criteria on pull versus push endoscopy. A Normal distribution curve is superimposed.

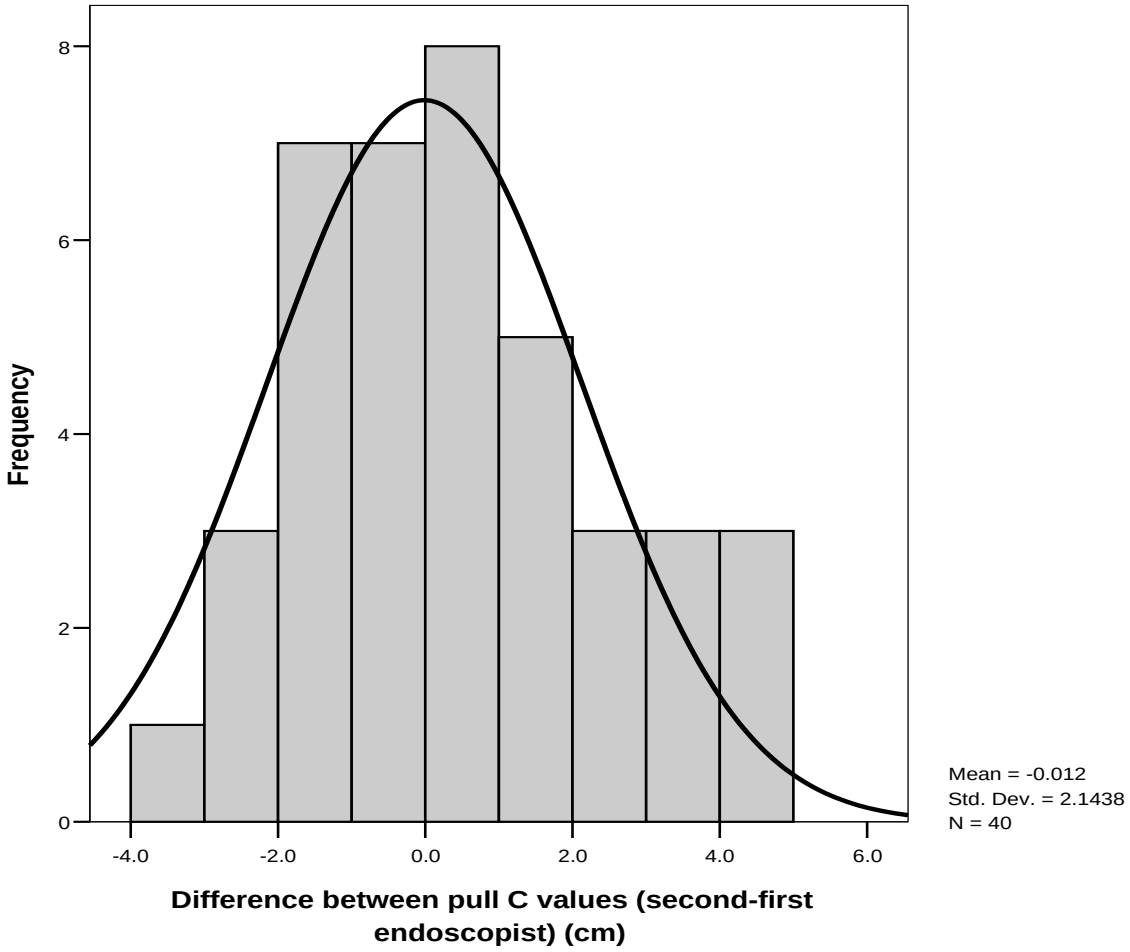


Figure 3.6 Distribution of the differences in M value measurements in 40 cases of Barrett's oesophagus using the Prague criteria on pull versus push endoscopy. A Normal distribution curve is superimposed.

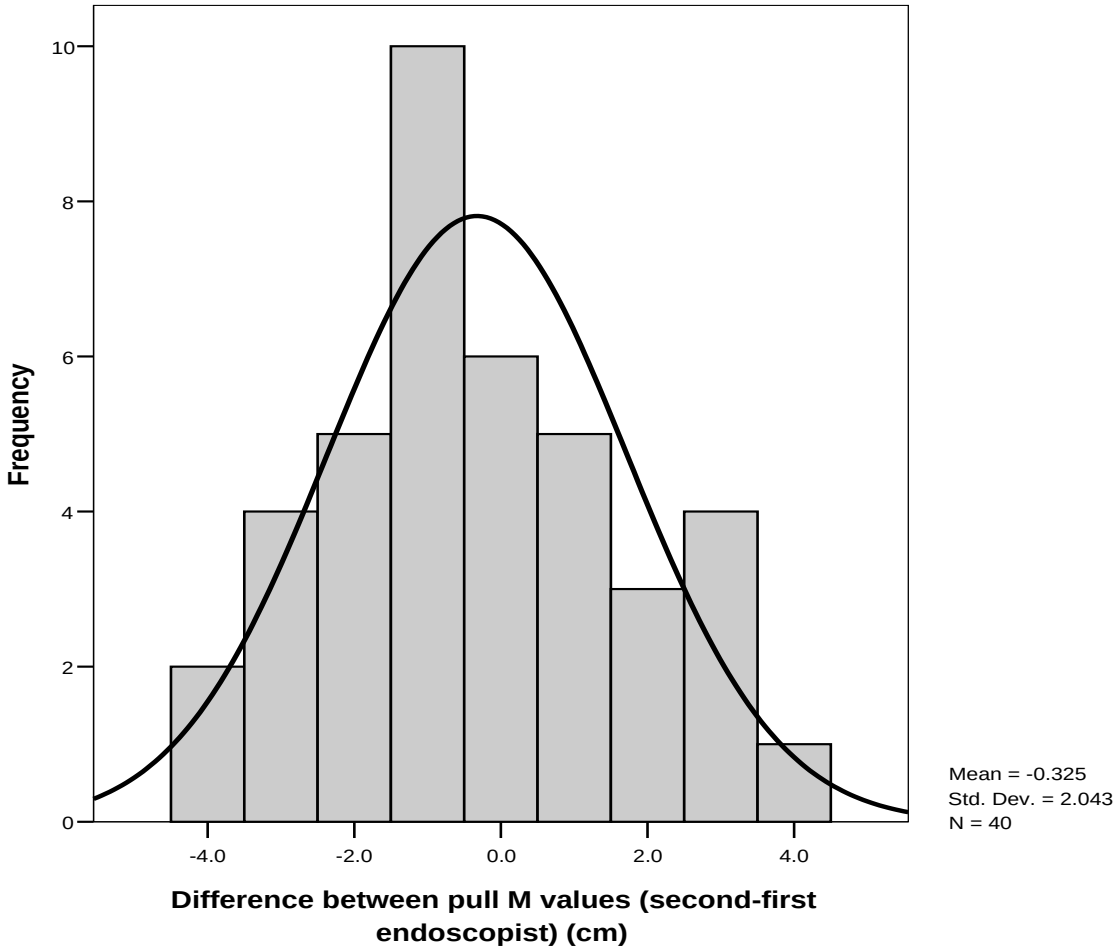


Figure 3.7 Distribution of the differences in C value measurements in 40 cases of Barrett's oesophagus using the Prague criteria, first versus second endoscopist on pull endoscopy. A Normal distribution curve is superimposed.

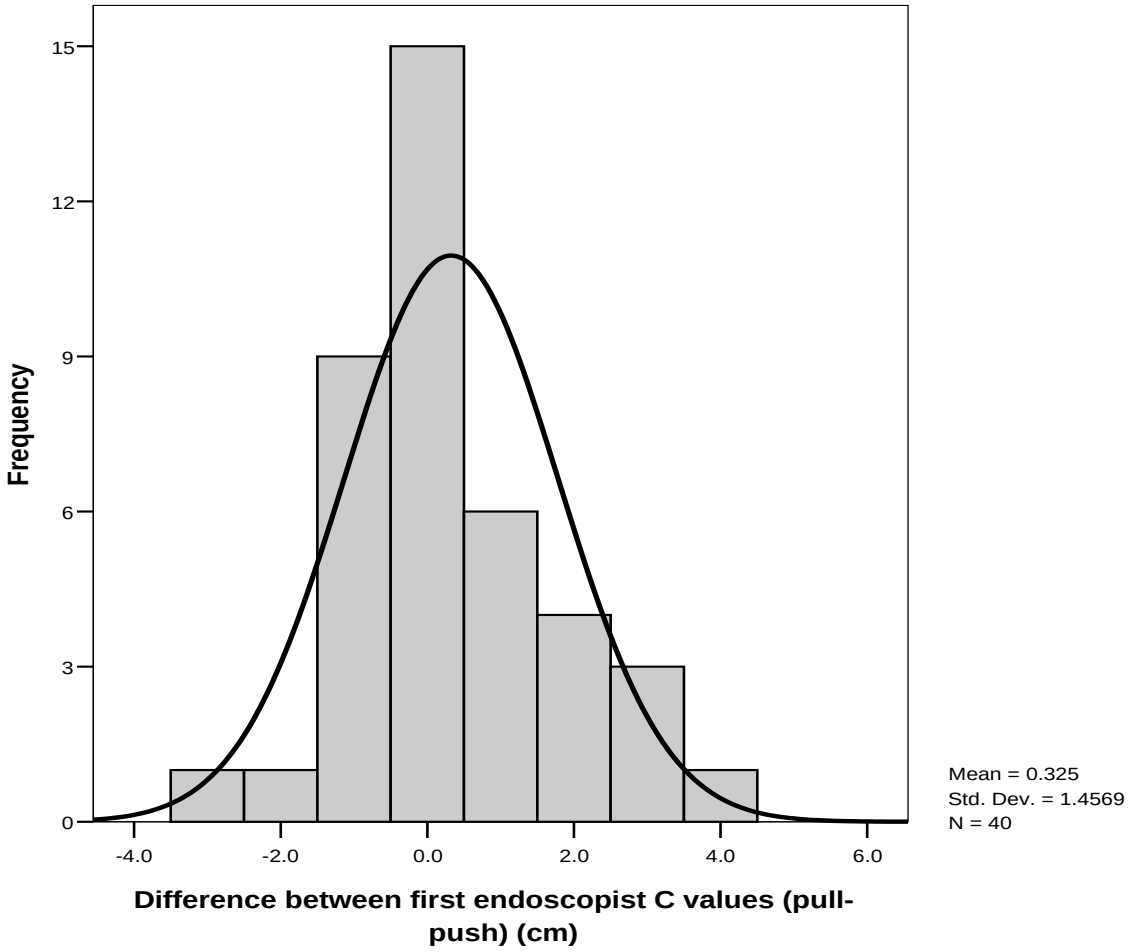


Figure 3.8 Distribution of the differences in M value measurements in 40 cases of Barrett's oesophagus using the Prague criteria, first versus second endoscopist on pull endoscopy. A Normal distribution curve is superimposed.

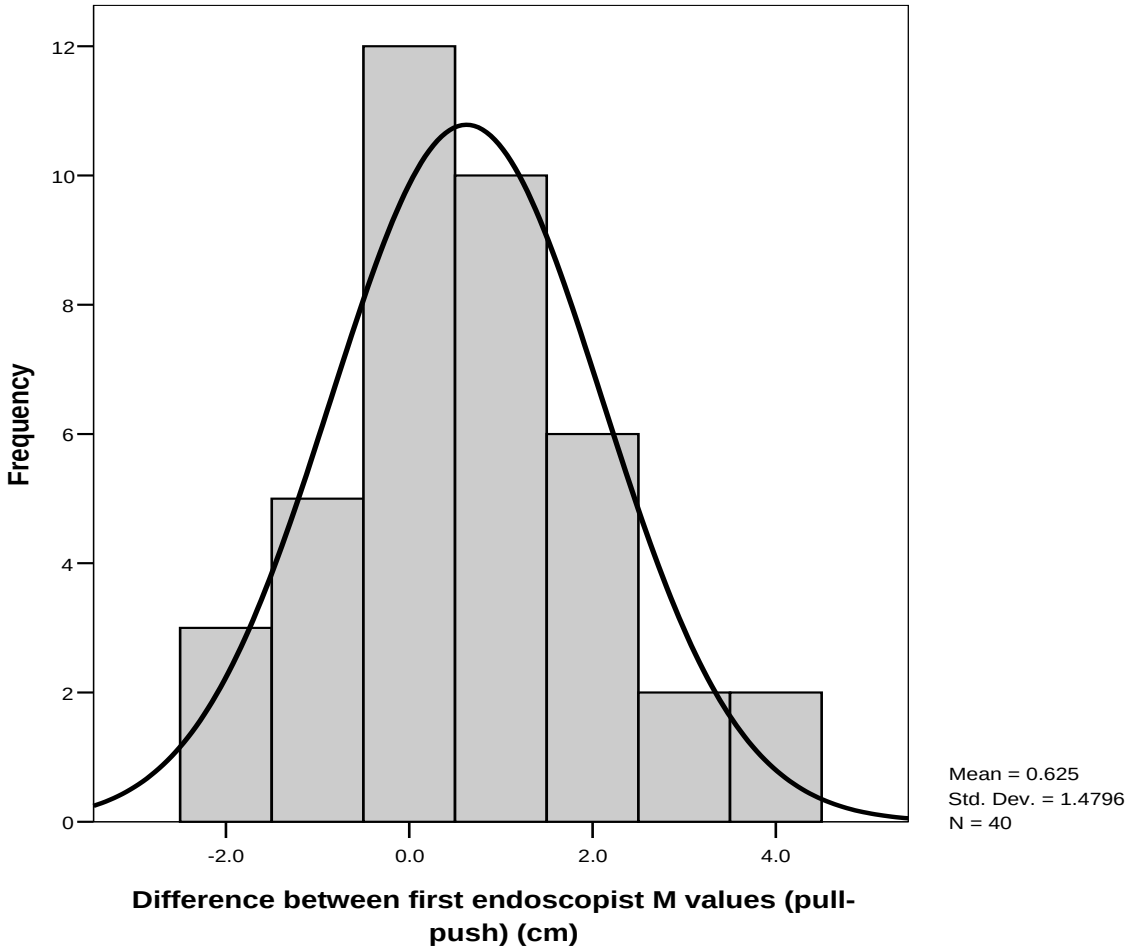


Figure 3.9 Difference against mean for C values in 40 cases of Barrett's oesophagus using the Prague criteria on pull versus push endoscopy. The mean differences +/- 2 standard deviations (SD) are indicated.

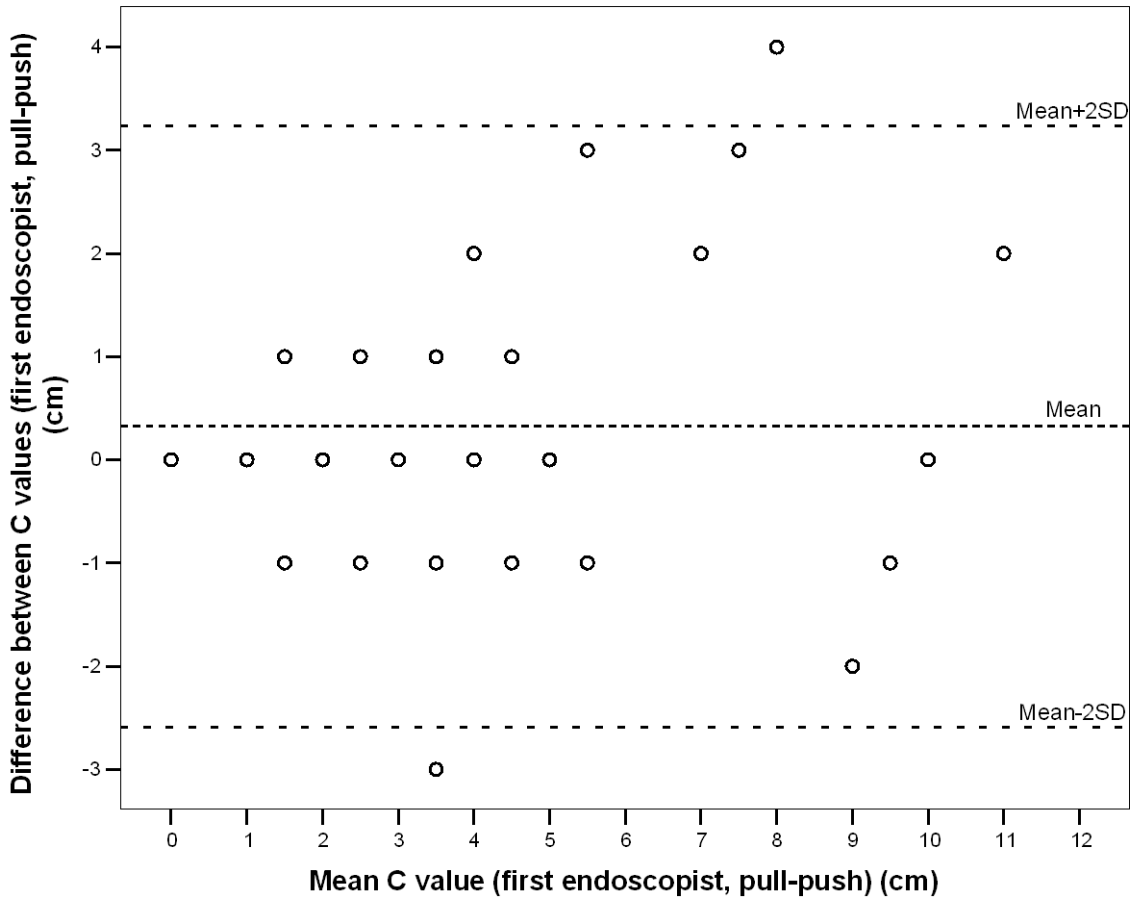


Figure 3.10 Difference against mean for M values in 40 cases of Barrett's oesophagus using the Prague criteria on pull versus push endoscopy. The mean differences +/- 2 standard deviations (SD) are indicated.

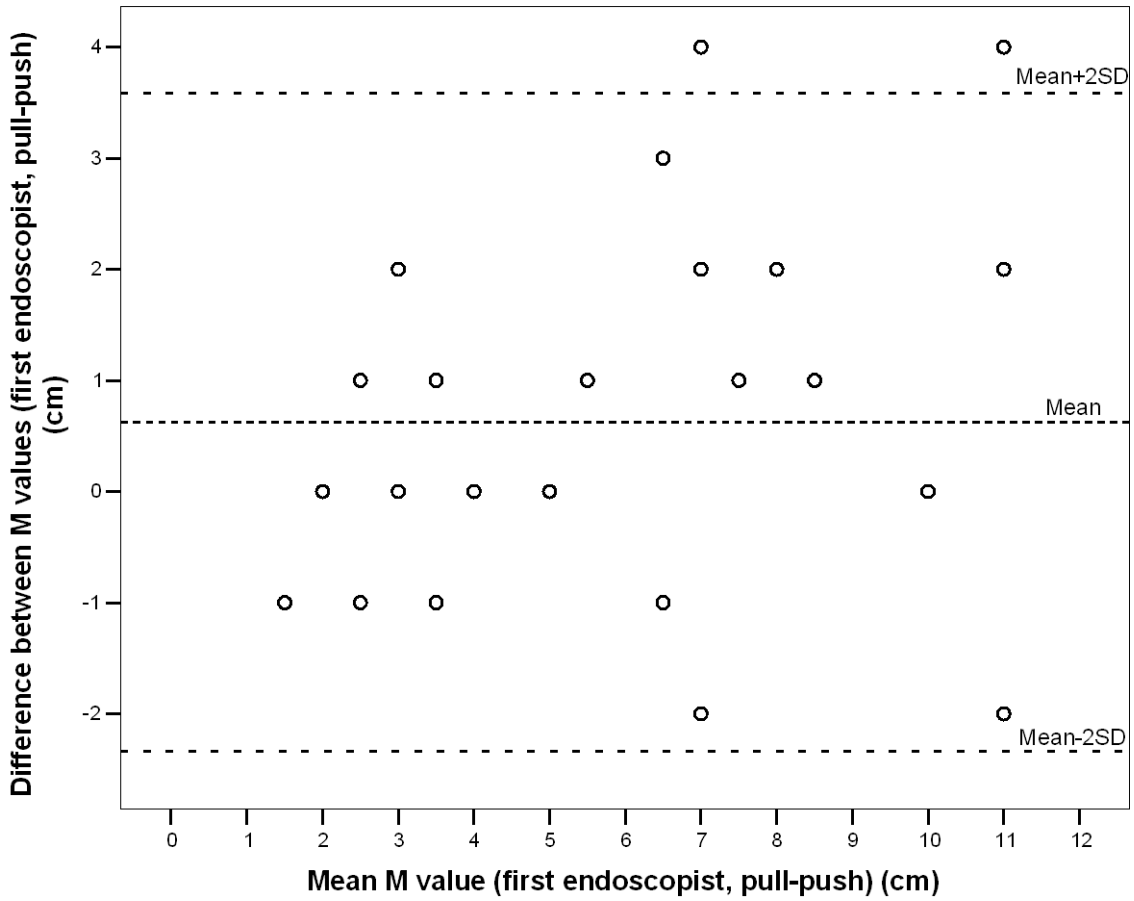


Figure 3.11 Difference against mean for C values in 40 cases of Barrett's oesophagus using the Prague criteria, first versus second endoscopist on pull endoscopy. The mean differences +/- 2 standard deviations (SD) are indicated.

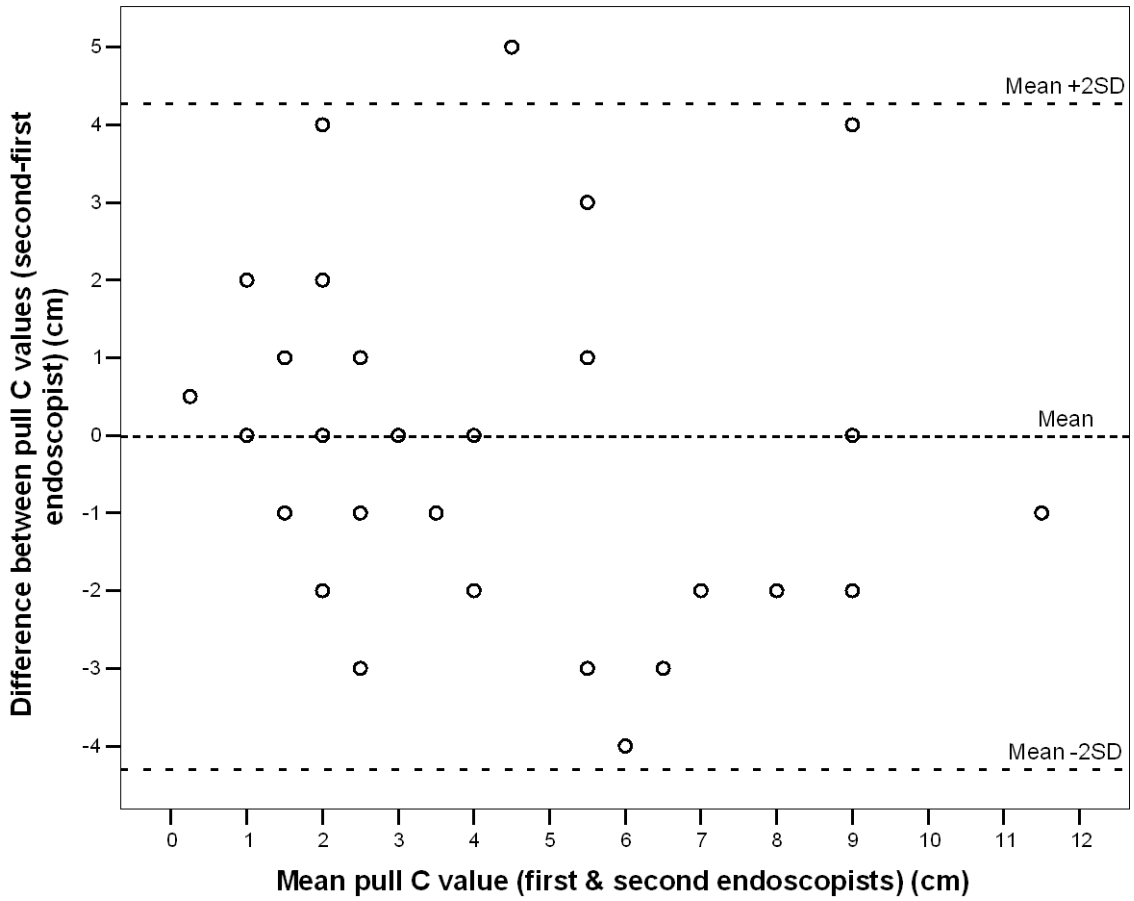


Figure 3.12 Difference against mean for M values in 40 cases of Barrett's oesophagus using the Prague criteria, first versus second endoscopist on pull endoscopy, C and D respectively. The mean differences +/- 2 standard deviations (SD) are indicated.

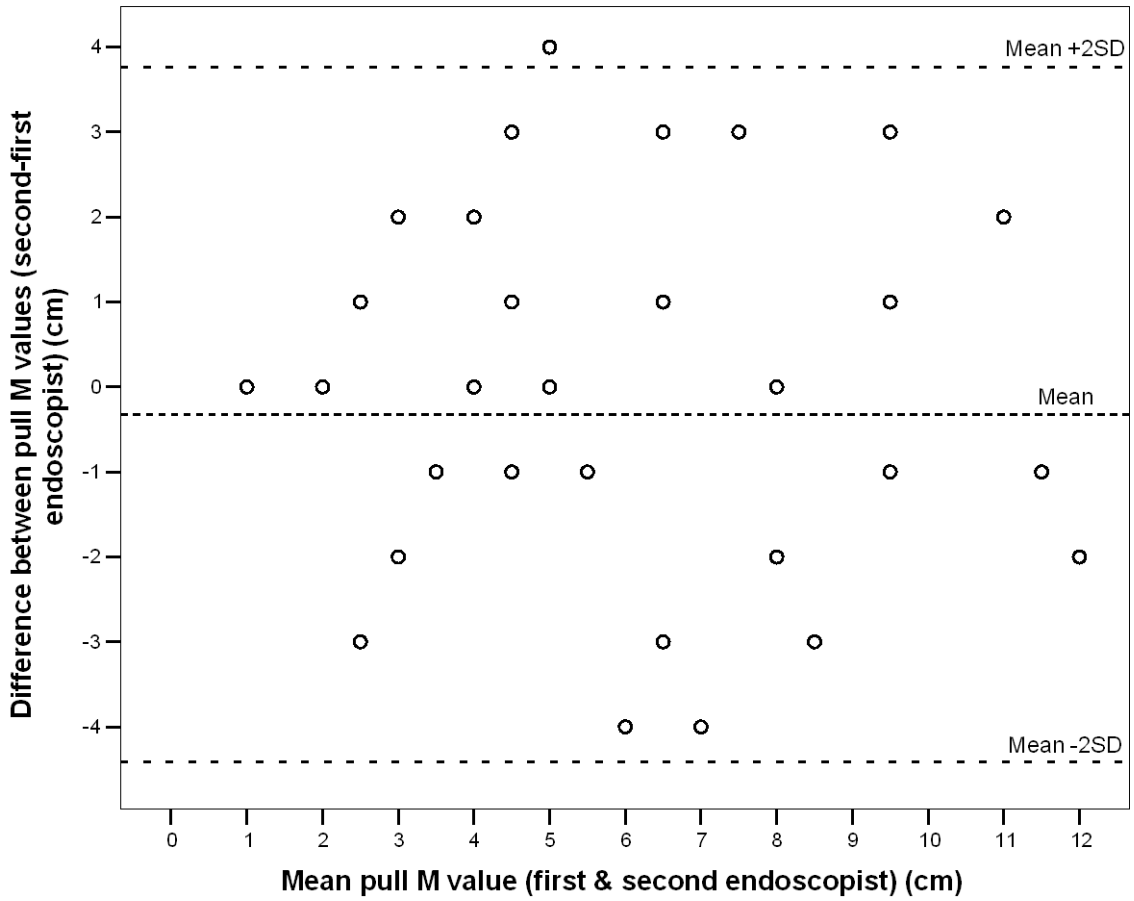


Table 3.5 Reliability coefficients (intraclass correlation coefficients) for recognizing different lengths of Barrett's oesophagus for pull compared to push endoscopy, and between endoscopists (inter-observer), subdivided by the presence or absence of a hiatus hernia.

	Reliability coefficient, pull versus push (95% confidence interval)		Reliability coefficient, inter-observer (95% confidence interval)	
	Cases with hiatus hernia (n=21)	Cases without hiatus hernia (n=19)	Cases with hiatus hernia (n=24)	Cases without hiatus hernia (n=16)
C value	0.88 (0.73-0.95)	0.86(0.68-0.94)	0.77 (0.53-0.89)	0.74 (0.40-0.90)
M value	0.88 (0.72-0.95)	0.84(0.63-0.94)	0.76 (0.52-0.89)	0.78 (0.47-0.92)

Table 3.6 Agreement by case and percentage for C & M values for push versus pull endoscopy and between endoscopists (inter-observer), subdivided by the presence or absence of a hiatus hernia.

	Agreement, push versus pull, case number (cumulative %)		Agreement, inter-observer, case number (cumulative %)	
	Cases with hiatus hernia (n=21)	Cases without hiatus hernia (n=19)	Cases with hiatus hernia (n=24)	Cases without hiatus hernia (n=16)
Exact agreement: C	8 (38)	7 (37)	4 (17)	3 (19)
1 cm difference: C	15 (71)	15 (79)	13 (54)	7 (44)
2 cm difference: C	19 (90)	16 (84)	19 (79)	11 (69)
3-4 cm difference: C	21 (100)	19 (100)	23 (98)	16 (100)
Exact agreement: M	5 (24)	7 (37)	4 (17)	2 (13)
1 cm difference: M	13 (62)	14 (74)	11 (46)	10 (63)
2 cm difference: M	20 (95)	16 (84)	18 (75)	11 (69)
3-4 cm difference: M	21 (100)	19 (100)	24 (100)	16 (100)

Table 3.7 Mean difference with limits of agreement (Bland-Altman analysis), between lengths of Barrett's oesophagus for pull compared to push endoscopy, subdivided by the presence or absence of a hiatus hernia.

	Push versus pull Cases with hiatus hernia (n=21)			Push versus pull Cases without hiatus hernia (n=19)		
	Mean difference (95% CI) (cm)	Lower limit of agreement, mean-2SD (95% CI)	Upper limit of agreement, mean+2SD (95% CI)	Mean difference (95% CI) (cm)	Lower limit of agreement, mean-2SD (95% CI)	Upper limit of agreement, mean+2SD (95% CI)
C value	0.19 (-0.51 to 0.89)	-2.88 (-4.09 to -1.67)	3.26 (2.05 to 4.47)	0.47 (-0.19 to 1.14)	-2.30 (-3.46 to -1.15)	3.25 (2.10 to 4.41)
M value	0.48 (-0.22 to 1.17)	-2.60 (-3.81 to -1.39)	3.55 (2.34 to 4.76)	0.79 (0.10 to 1.48)	-2.08 (-3.28 to -0.89)	3.66 (2.47 to 4.86)

Table 3.8 Mean difference with limits of agreement (Bland-Altman analysis), between lengths of Barrett's oesophagus between endoscopists (inter-observer), subdivided by the presence or absence of a hiatus hernia.

	Inter-observer Cases with hiatus hernia (n=24)			Inter-observer Cases without hiatus hernia (n=16)		
	Mean difference (95% CI) (cm)	Lower limit of agreement mean-2SD (95% CI)	Upper limit of agreement mean+2SD (95% CI)	Mean difference (95% CI) (cm)	Lower limit of agreement mean-2SD (95% CI)	Upper limit of agreement mean+2SD (95% CI)
C value	0.29 (-0.58 to 1.17)	-3.86 (-5.37 to -2.34)	4.44 (2.93 to 5.95)	-0.47 (-1.65 to 0.71)	-4.93 (-6.98 to -2.88)	3.99 (1.95 to 6.04)
M value	-0.04 (-0.93 to 0.85)	-4.27 (-5.82 to -2.73)	4.19 (2.65 to 5.73)	-0.75 (-1.76 to 0.26)	-4.58 (-6.34 to -2.82)	3.08 (1.32 to 4.84)

3.4 Discussion

The Prague C & M criteria were presented as a simple, reliable consensus-based endoscopic classification system to describe and classify endoscopic Barrett's oesophagus (Sharma et al. 2006). They were developed with extensive internal and external validation by trained endoscopists with an interest in Barrett's oesophagus. The authors acknowledge the limitations of using video clips over a live endoscopy setting. Whilst this allowed data to be recorded by multiple endoscopists, the assessors focused solely on the video screen (with freeze-frame available) without all the other concerns of live endoscopy. The distance from the incisors was displayed in the top corner of the screen, obviating the need to glance down at the endoscope, with possible unintentional movement of the scope. The original findings have been strengthened by the recently published study by Lee *et al*, as part of the Asian Barrett's Consortium (Lee et al. 2010). This study involved one of the original authors, Dr P Sharma, and was methodologically identical, although used 34 assessors studying 21 video clips. Despite the uncommon occurrence of Barrett's oesophagus in Asia, there was excellent agreement in the diagnosis and grading of Barrett's oesophagus using the Prague criteria. The reliability coefficients for C, M, and the proximal margin of gastric folds were; 0.92, 0.94 and 0.86 respectively. They confirmed the low inter-observer reliability in recognizing Barrett's oesophagus <1 cm in length, with reliability coefficients for C and M of 0.18 and 0.21 respectively.

To address these issues, I present the first validation of the Prague C & M criteria in a live endoscopy setting. With each endoscopist's measurements being recorded serially, two experienced endoscopists could participate per patient. Independent calculation of the C & M values removed any error on the endoscopists' behalf, making this a study of agreement in landmark interpretation and measurement. The intra- and inter-observer agreements for the position of the proximal margin of the gastric folds were high, with reliability coefficients of 0.85 and 0.78 respectively. It must be remembered that the measurement of this landmark is a specific distance that will change due to

oesophageal motility, respiration and patient movement. This contrasts with the C & M values that are a difference between two landmarks, designed to overcome this exact problem. The Pearson correlation coefficients for pull versus push endoscopy and between endoscopists demonstrate the strong relationship between these variables, although the distribution of data away from the line of equality in Figure 3.1-4 point to the lack of perfect agreement. The reliability coefficients for intra- and inter-observer measurements of C & M do suggest "almost perfect" reliability at best, although with only "substantial" reliability between endoscopists at pull endoscopy (with reliability coefficients for C & M of: 0.88 and 0.87 for intra-observer; 0.75 and 0.76 for inter-observer) (Table 3.2). This contrasts with the almost perfect reliability observed in the validation study (reliability coefficients for C & M of 0.94 and 0.93) (Sharma et al. 2006) However the percentage agreement between pair wise comparisons was similar to the original study for push against pull endoscopy, differing at most by 2 cm in 87% and 90% of cases for C & M respectively (compared to 97% and 95%). This fell to 75% and 73% for inter-observer measurements, with only 18% and 15% of C & M values agreeing exactly (Table 3.3).

The agreement between the two endoscopists was further analyzed by the Bland-Altman approach (Figure 3.9-12). This avoids the potentially misleading implications of the Pearson correlation coefficient, which measures the strength of a relation between two variables, not the agreement between them. The greatest difference between C & M values was only 0.63 cm (Table 3.4), but it is the wide limits of agreement that raise concern. The differences in measurement between the endoscopists are normally distributed, and 95% will therefore lie within approximately two standard deviations either side of the mean (the limits of agreement). Therefore the first endoscopist's C & M values may be approximately 4 cm above or below the second endoscopist's C & M values. Clearly this would be clinically unacceptable.

The "almost perfect" reliability of the intra-observer C & M values supports the original internal validation finding that there was minimal difference in recording the length of

Barrett's at push or pull endoscopy (after gastric deflation), with correspondingly narrower limits of agreement on Bland-Altman analysis. Interestingly, the pull values were consistently greater than the corresponding push values. This suggests that despite gastric deflation, the proximal margins of the gastric folds are pulled down into the abdominal cavity after push endoscopy by remaining air in the stomach. Our findings support the suggestion that the criteria are easy to learn and apply by individual endoscopists. There was a consistent reduction in reliability and agreement from intra-observer to inter-observer values for C & M. These differences between endoscopists were presumably due to landmark interpretation or movement: respiratory, cardiac, or contraction of the oesophagus itself.

No clinically significant differences in agreement of the C & M values in the presence or absence of a hiatus hernia were observed, either for push versus pull endoscopy or inter-observer. This supports the validity of measuring the distance between two landmarks; movement will alter the exact values but the difference between them should be constant. If the degree of herniation of the stomach into the thoracic cavity is relatively fixed, then this should not have a major impact on the agreement measured C & M values.

The first study to provide data on the consistency with which Barrett's oesophagus is demonstrated on different endoscopic examinations in the same patient concluded that there was substantial variability in the results of closely spaced tests for Barrett's oesophagus (Kim et al. 1994). The mean endoscopic measurements of the lower oesophageal sphincter and the proximal level of Barrett's oesophagus on two endoscopies performed in 111 patients by the same endoscopist 6 weeks apart were constant. However, in 9% of patients the values for both measures differed by $\geq$4 cm, a result in keeping with our findings. No comment is made as to the impact of these landmark changes on the length of the Barrett's oesophagus, if changes in both measures within a given patient were of the same magnitude and in the same direction, the length of Barrett's oesophagus would be unchanged. However, 18% of

patients meeting the endoscopic criteria for Barrett's oesophagus in use at that time did so in only one of the two closely spaced procedures, implying an apparent shortening of the observed length of Barrett's oesophagus.

A study from Japan used endoscopic photographs to assess whether the distal end of the lower oesophageal palisade vessels or the proximal margin of the gastric folds resulted in a more consistent diagnosis of Barrett's oesophagus (Amano et al. 2006). The reliability coefficients were unacceptably low at 0.16 and 0.12 respectively. The study was repeated with a detailed explanation of the Prague C & M criteria for 25 endoscopists, and the reliability coefficient increased from 0.17 to 0.35, an outcome comparable with our results. Moreover, Guda *et al* designed a study to determine the accuracy and intra- and inter-observer variability between 12 endoscopists in the measurement of length of Barrett's oesophagus with an endoscope in a model oesophagus (Guda et al. 2004). The mean difference between true length and measured length was 1.1 cm, and the κ statistic for intra-observer agreement was 0.4, with 16% of measurements defined as accurate (values differed by 0.5 cm at most from the correct value). These results are consistent with our findings, with a similar mean difference in measurements and percentage pair wise agreement. With respect to the range of measurements of Barrett's oesophagus, the authors give an example for a true length of 7 cm, where values were reported that ranged from 4 to 14cm. This is entirely consistent with our Bland-Altman analysis (Figure 3.9-12).

The most comparable study methodologically reported on 55 patients with Barrett's oesophagus in whom two endoscopies were performed by the same endoscopist (mean follow up 18.6 months) and 40 patients in whom two endoscopies were performed by two different endoscopists (mean follow up 15.7 months) (Dekel et al. 2003). Measurement of the length of Barrett's oesophagus was taken from the proximal margin of continuous Barrett's oesophagus to the proximal margin of the gastric folds or to the end of the tubular oesophagus, akin to the Prague classification C value. The absolute mean difference between the first and second endoscopies was 1.6 cm and

1.4 cm for measurements made by the same and by different endoscopists respectively. However, no comment is made as to the limits of agreement. The greatest mean difference in our study was 0.63 cm, suggesting an even greater degree of agreement, although the wide limits of agreement must not be forgotten. Furthermore, the longer the segment of Barrett's oesophagus, the greater the absolute difference in Barrett's oesophagus length between the consecutive endoscopies. This effect is seen in our study for pull compared to push endoscopy, with the scatter of the differences increasing as the mean C & M values increase (Figure 3.9-12). The authors propose a "range of variability" of up to 1.6 cm when measuring Barrett's oesophagus length, with only a greater extent of regression or progression considered to represent a true change in the length of Barrett's oesophagus.

As discussed above, there is conflicting evidence that aggressive acid suppression with proton pump inhibitors promotes regression of Barrett's oesophagus. Several studies have addressed this issue, although with different study populations, study design and type and dosage of PPI. Studies of 13-68 patients with 2-3 endoscopists resulted in no significant change to a mean reduction in Barrett's oesophagus length of 2.6cm (Gore et al. 1993;Malesci et al. 1996;Sharma et al. 1997;Peters et al. 1999;Wilkinson et al. 1999). Our findings suggest that a proportion of these changes in Barrett's oesophagus length will be due to intra- or inter-observer variability. Our findings support the concept of a regression/progression threshold, and suggest that any claim of a change in the length of Barrett's oesophagus made by future studies using the C & M classification needing to be $\geq$0.63 cm (Dekel et al. 2003). However, the confidence in this measure of agreement lies outside of acceptable clinical practice, and we would not consider this to be applicable at present. Larger studies will be able to address this degree of variability.

The limitation of this study was the small number of assessors. This was a practical necessity given the live endoscopy setting of the study. The reliability of endoscopists to measure shorter lengths of Barrett's oesophagus (<1 cm) in the validation study was

understandably reduced. This factor was avoided in our unselected population of Barrett's oesophagus patients attending for surveillance endoscopy, with no cases of Barrett's oesophagus <1 cm in length. An undoubted source of error was the standard 5 cm markings on the endoscopes. This contrasts to the centimeter indicator on the video clips assessed by the experts in the original validation, and the need for the endoscopist to look away from the screen to the scope, with possible inadvertent movement of the scope. The presence of markings at 1 cm (or smaller) can only help to improve the accuracy of measurement and improve agreement. We would add our support to encourage endoscope manufacturers to include this as a standard feature in endoscope design (Guda et al. 2004;Sharma et al. 2006). To overcome any difference in measurements made by the second endoscopist due to the presence of air insufflated by the first endoscopist, a future study would have to compare push endoscopy only. The patient would have to attend for endoscopy twice, ideally with as short an interval as possible.

In conclusion, the agreement of C & M values in cases of Barrett's oesophagus between two endoscopists in a live endoscopy setting is consistent with the degree of agreement reported when the Prague C&M criteria were originally presented. However, detailed analysis of the limits of agreement suggests a clinically unacceptable measurement error. We found the criteria easy to use and quick to apply, and support the standardization of reporting to improve documentation in individual patients, and patient classification in clinical trials. However, the inherent variability of measuring the length of Barrett's oesophagus described in this study must always be considered, especially in trials of treatments that report a reduction in the length of Barrett's oesophagus.

3.4.1 Future work

The methodology employed in this study will always mean the study of agreement of the Prague criteria will be limited to a maximum of two endoscopists. It is difficult to

consider any alternative approach, but an increased number of patients would improve the power of the study. Ideally, pairs of endoscopists in multiple centres would be the best way forward.

As mentioned above, to overcome any difference in measurements made by the second endoscopist due to the presence of air insufflated by the first endoscopist, a future study could compare push endoscopy only, with two endoscopic procedures.

The measurement of any landmark is a vital basic skill in endoscopy, particularly in the oesophagus. It seems unlikely that any advanced technology such as chromoendoscopy, image-enhanced endoscopy, or zoom magnification endoscopy will improve the inter-observer agreement of the Prague criteria, but may improve the diagnosis and agreement of short sections of Barrett's oesophagus measuring <1 cm in length (Goda et al. 2007;Ngamruengphong et al. 2009).

Any future studies will be greatly enhanced by the provision of centimeter markings on future endoscopes.

4 EXPRESSION AND ACTIVITY OF THE LEUKOTRIENE PATHWAY IN BARRETT's METAPLASIA, DYSPLASIA AND OESOPHAGEAL ADENOCARCINOMA

4.1 Introduction

There is increasing interest in the role played by arachidonic acid metabolites in Barrett's oesophagus, with a large prospective multi-centre interventional study (AspECT) in progress to see whether suppression of COX by aspirin can reduce the progression of Barrett's metaplasia to dysplasia and cancer (2010b). Recent work has shown that COX-2 expression plays a significant role in the progression of Barrett's metaplasia to dysplasia and carcinoma (Shirvani et al. 2000;Bhandari et al. 2001). Lipoxygenase expression has been well documented in many solid tumour cells, including prostate, colon, stomach, and oesophagus (Hong et al. 1999;Hoque et al. 2005;Chen et al. 2004). Many lipoxygenase products have been linked to tumorigenesis in *in vitro* and *in vivo* models, and early studies of lipoxygenase inhibitors suggested anti-tumorigenic effects (Hussey & Tisdale 1994;Steele et al. 1999). Leukotriene products of 5-lipoxygenase have recognised pro-inflammatory, mucus secretagogue and microvascular actions relevant to Barrett's oesophagus. There is further concern that as NSAIDs act to remove PGE_2, in doing so they potentiate the deleterious effect of LTs by removing the brake on LT synthesis (the so-called "PGE_2 brake" hypothesis) or by shunting their common precursor, arachidonate, down the leukotriene pathway (the "shunting" hypothesis) (Sampson & Holgate 2004;Szczeklik 1990;Szczeklik 1995;Sestini et al. 1996).

There are limited data on the role played by leukotrienes on the Barrett's metaplasia-dysplasia-adenocarcinoma sequence progression, with no published data quantifying leukotriene concentration in human oesophageal tissues. Increasing expression of 5-LO by immunohistochemistry through normal, Barrett's, dysplastic mucosa and adenocarcinoma was observed in one series (Chen et al. 2004). In another study, 5-LO was expressed in the majority of both oesophageal squamous carcinomas and adenocarcinomas, as well as in a range of oesophageal cell lines. 5-LO inhibitors produced an increase in apoptosis, associated with reduced levels of LTB_4 (Hoque et al. 2005). In a rat model of oesophageal adenocarcinoma, increased levels of 5-, 8-, 12-,

15-HETE, LTB_4, and PGE_2 compared to the proximal oesophagus were found by mass spectrometry (Chen et al. 2002). Two studies utilising the same model observed a reduced incidence of adenocarcinoma with both broad (sulindac, NDGA) and selective (celecoxib, zileuton) inhibitors of COX-2 and 5-LO respectively (Chen et al. 2002;Chen et al. 2004).

The key aim of the studies in this chapter was to investigate the activity of the leukotriene pathway and the expression of 5-LO, its activating protein FLAP, and COX-2 in Barrett's metaplasia, dysplasia and adenocarcinoma.

4.2 Methods

4.2.1 Immunohistochemistry

Immunohistochemistry was performed as described in section 2.1 to assess cellular immunostaining for COX-2, 5-LO, and FLAP in anonymous archival paraffin-embedded sections of Barrett's metaplasia, low- and high-grade dysplasia, and adenocarcinoma. This work was kindly arranged by Prof I Cree of the Translational Oncology Research Centre, and Dr P Bhandari, Department of Gastroenterology, Queen Alexandra Hospital, Portsmouth. The samples were selected from the hospital archives by a single pathologist, Dr D Poller, and analysed by Mrs P Johnson, within the remit of a generic LREC approval held by the department, and specific LREC approval obtained by Dr P Bhandari. Negative controls were obtained by omitting the primary antibody, and positive controls used tonsil (COX-2) and nasal polyp (5-LO) sections. Sections were scored by myself and Dr Poller semi-quantitatively, on the basis of intensity of staining and proportion of positive cells (total score between 0-300), a technique that has been previously validated in oesophageal adenocarcinoma sections (Bhandari et al. 2006; Bhandari et al. 2005). An intensity score is given, with 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for intense staining. This is multiplied by the proportion score, the percentage of tissue staining to the maximal degree.

4.2.2 Oesophageal Biopsy Specimens

Anonymised oesophageal biopsy specimens from patients with Barrett's oesophagus, with and without dysplasia, and Barrett's associated oesophageal adenocarcinoma were obtained prospectively with informed consent from Southampton General Hospital under LREC approval (212/03/t, 08-H0501-3). With limited data regarding the optimal measurement of products of the leukotriene pathway, a method for extracting leukotrienes from whole endoscopic biopsy samples was formulated, as described in section 2.2. Initial studies were aimed at producing repeatable results

using progressively smaller amounts of tissue, to allow time- course and inhibition studies to confirm production of leukotrienes by oesophageal mucosal tissue.

4.3 Results

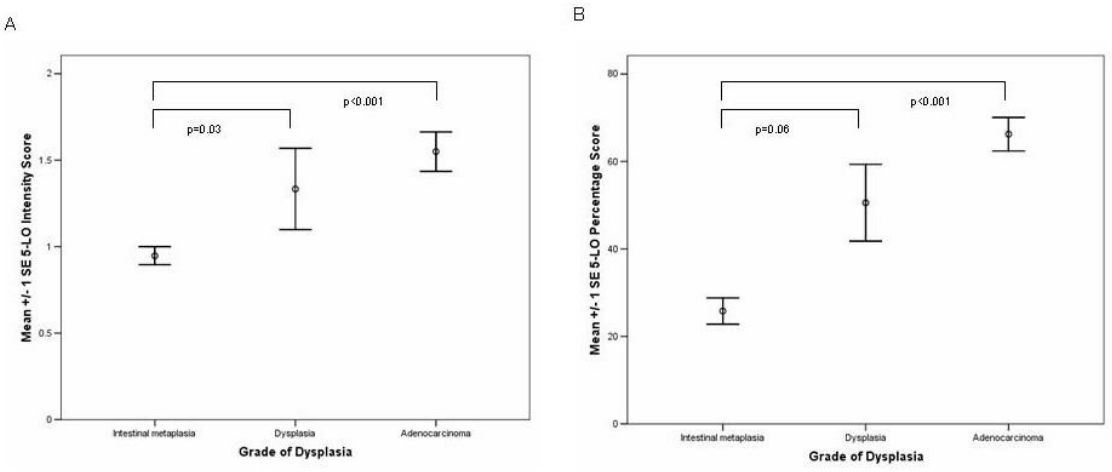
4.3.1 Immunohistochemistry

Anonymised archival specimens were obtained for 19 cases of Barrett's intestinal metaplasia, 9 cases of Barrett's with dysplasia (7 high grade dysplasia, 2 low grade dysplasia), and 20 cases of oesophageal adenocarcinoma. Positive staining was seen predominantly in the epithelial cells of all tissue types, but included some stromal and polymorphonuclear cells within blood vessels. The mean intensity proportion scores and 95% confidence intervals for 5-LO, FLAP and COX-2 are given in Table 4.1. Kruskal-Wallis testing revealed a significant difference across all tissue groups for 5-LO, FLAP and COX-2 ($p<0.002$). Pair wise comparisons were then made using the Mann-Whitney U-test, demonstrating significant differences in intensity proportion scores for both 5-LO and FLAP in dysplasia and adenocarcinoma compared to Barrett's oesophagus ($p<0.005$). There was a significant difference between COX-2 intensity proportion score for adenocarcinoma versus Barrett's oesophagus ($p<0.005$) and for adenocarcinoma versus dysplasia ($p<0.05$). These differences are illustrated in scatter plots in Figures 4.1, 4.2 and 4.3 for 5-LO, FLAP and COX-2 respectively.

Table 4.1 Mean intensity proportion scores, standard error and 95% confidence interval for 5-LO, FLAP and COX-2 immunostaining. The table summarises the individual data illustrated in Figures 5.1, 5.2 and 5.3.

	Grade of Dysplasia	N	Mean	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
5-LO	Intestinal metaplasia	19	25.8	3.0	19.5	32.1
	Dysplasia	9	81.1	19.7	35.7	126.5
	Adenocarcinoma	20	105.3	11.3	81.7	128.8
FLAP	Intestinal metaplasia	19	40.3	7.0	25.6	55.0
	Dysplasia	9	112.8	22.0	62.0	163.6
	Adenocarcinoma	20	109.3	14.3	79.2	139.3
COX-2	Intestinal metaplasia	18	9.7	4.2	0.8	18.6
	Dysplasia	9	28.3	20.5	-18.9	75.5
	Adenocarcinoma	19	74.7	16.4	40.3	109.2

Figure 4.1 Mean intensity and percentage scores (A, B) and their product intensity immunostaining score (C, bars represent mean) for 5-LO by metaplasia, dysplasia and adenocarcinoma.



C

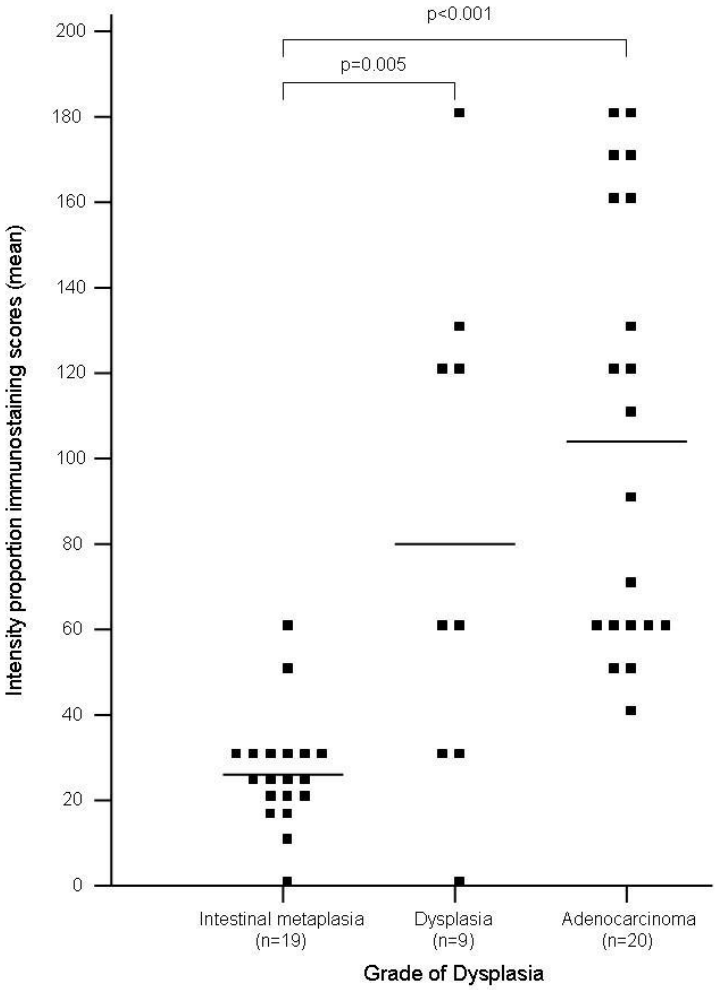
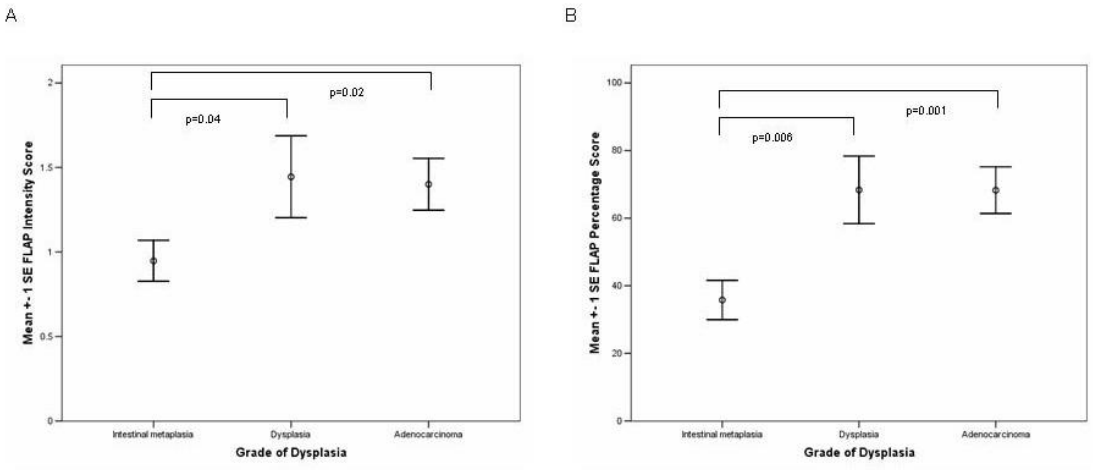


Figure 4.2 Mean intensity and percentage scores (A, B) and their product immunostaining score (C, bars represent mean) for FLAP by metaplasia, dysplasia and oesophageal adenocarcinoma.



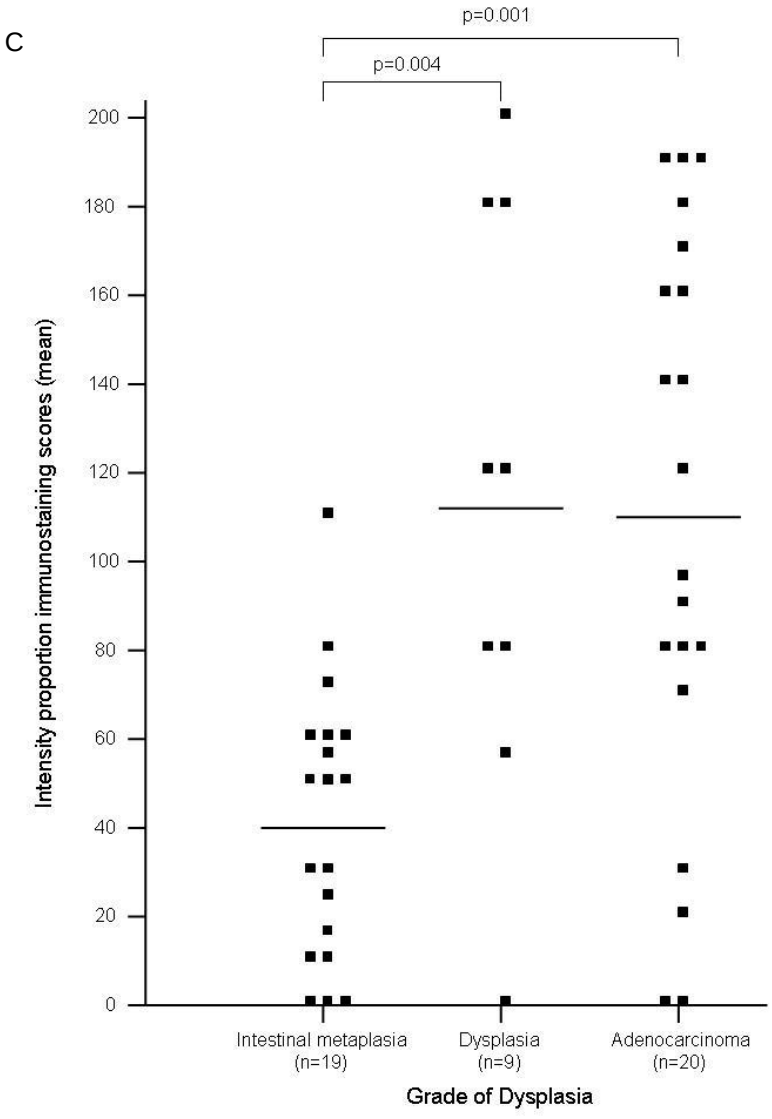
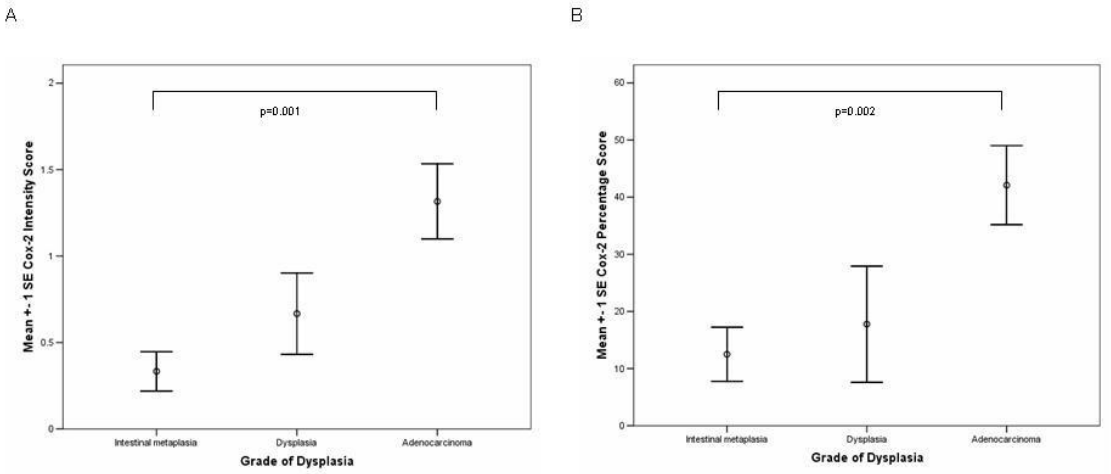
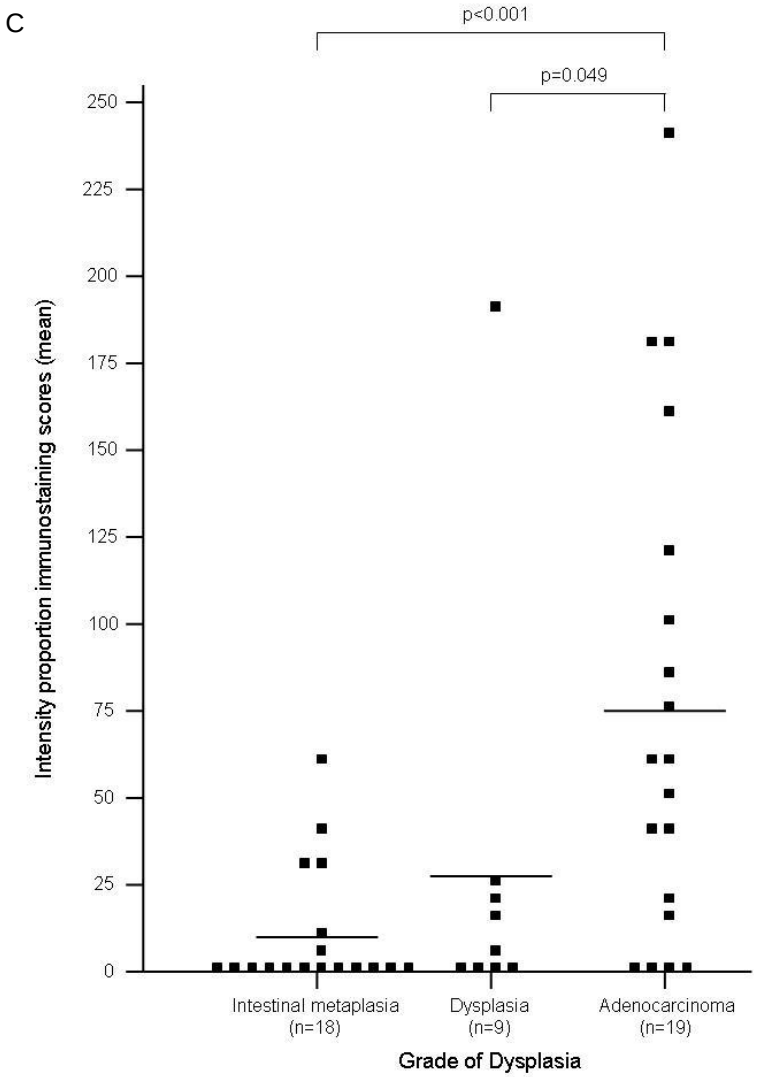


Figure 4.3 Mean intensity and percentage scores (A, B) and their product immunostaining score (C, bars represent mean) for COX-2 by metaplasia, dysplasia and oesophageal adenocarcinoma.





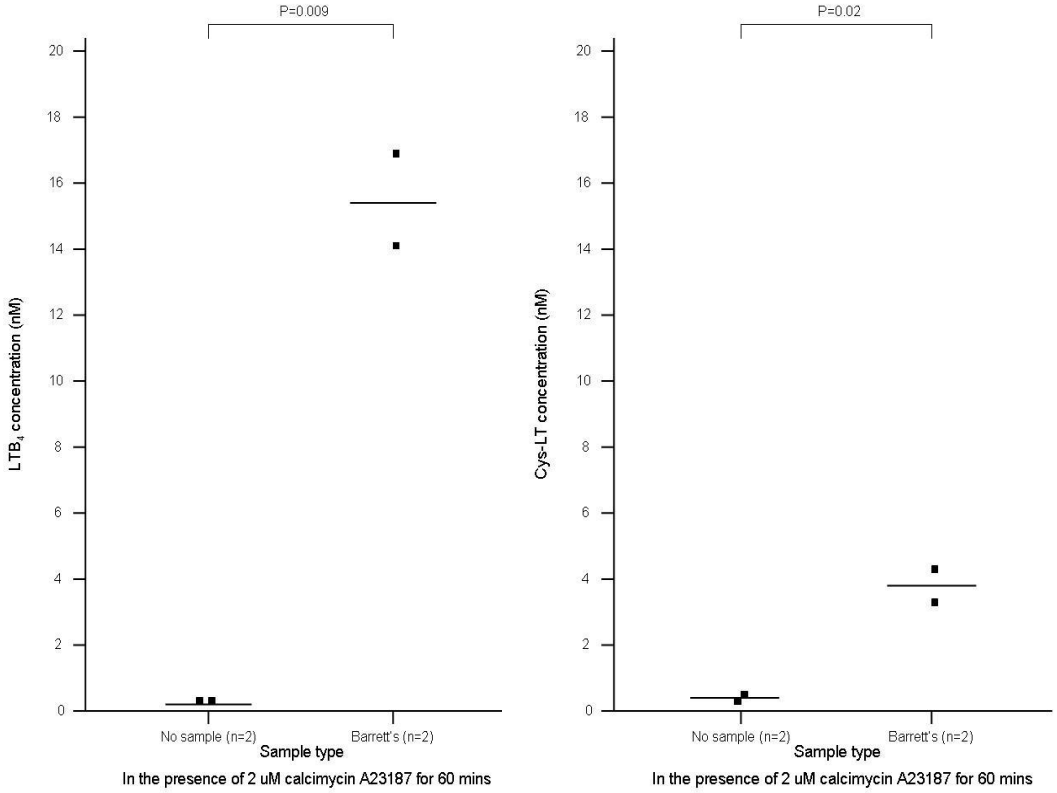
4.3.2 Quantification of LTB_4 and Cys-LT concentrations from whole biopsy specimens by EIA

For all the studies in this chapter, a total of 40 patients were enrolled; 31 male patients (mean age 64 years, range 34-85), and 9 female patients (mean age 66, range 57-76). One patient was taking low dose aspirin, two took non-steroidal anti-inflammatory drugs (NSAID) as required, and three were not taking a proton pump inhibitor (PPI). There was no significant difference between sex, age or drug use and LTB_4 or cys-LT concentration. There was no correlation between the maximal length of Barrett's oesophagus and LTB_4 or cys-LT concentration.

In the explant/immunoassay experiments, freshly-obtained oesophageal biopsies from each subject group were equilibrated in DMEM for 5 minutes before culture for a further 60 minutes in the presence or absence of calcium ionophore A23187 (2 uM). After methanolic extraction, evaporation and reconstitution in immunoassay buffer, the generated leukotrienes were quantified per mg of wet tissue weight using highly-sensitive enzyme immunoassays for LTB_4 and for total cysteinyl-leukotrienes ($LTC_4/D_4/E_4$) (Cayman Chemicals Inc).

In a preliminary control experiment excluding the biopsy tissue, incubation of DMEM alone for 60 min with 2 uM A23187 resulted in very low levels of LTB_4 being detectable (0.2 nM +/- 0.03; n=2). In contrast, identical cultures with Barrett's oesophagus tissue included and stimulated with 2 uM A23187 resulted in mean LTB_4 levels after 60 min of 15.46 nM (+/-1.44; n=2) (Figure 4.4). Corresponding cys-LT levels were 0.36 nM (+/- 0.07) without tissue and 3.67 nM (+/- 0.48, n=2) with tissue (Figure 4.4).

Figure 4.4 LTB_4 and Cys-LT concentrations at time 60 min, present in initial culture medium, determined by EIA after methanol extraction and centrifugal evaporation, in two samples of Barrett's oesophagus versus no sample, in the presence of A23187 (2 uM) (bars represent mean).



Having established the low levels of non-specific immunoreactivity for leukotrienes in the absence of tissue, and that Barrett's oesophagus biopsy tissue generates sufficient quantities of both LTB_4 and total cysteinyl-LTs when stimulated with A23187 (2 uM) for 60 min, these conditions were retained to compare leukotriene production in oesophageal tissue from the following groups:

(i) normal squamous oesophageal mucosa adjacent to Barrett's,

(ii) Barrett's oesophagus,

(iii) Barrett's adjacent to HGD or adenocarcinoma,

(iv) High grade dysplasia (HGD), and

(v) Adenocarcinoma.

Figures 4.5 and 4.6 show that there were no significant differences in either LTB_4 or cys-LT production between squamous tissue and Barrett's oesophagus, although LTB_4 levels appeared relatively high in three subjects in the Barrett's group. Kruskal-Wallis tests however showed that there were significant increases in production of LTB_4 ($p=0.02$) and cys-LTs ($p=0.03$) across the grades of dysplasia from (i) to (v). Significantly more LTB_4 was produced by adenocarcinoma tissue (411.6 +/-160.2 pg/mg) than by Barrett's oesophagus (63.0 +/-14.8 pg/mg, $p=0.004$) or squamous (36.5 +/-4.1 pg/mg, $p=0.001$). Significantly more cys-LT was produced by adenocarcinoma (230.7 +/-33.9 pg/mg) when compared with Barrett's oesophagus (110.5 +/-14.3 pg/mg, $p=0.001$) or squamous (107.1 +/-17.4 pg/mg, $p=0.002$).

Given the small number of cases in the Barrett's next to HGD/adenocarcinoma ($n=4$) and the HGD ($n=2$) groups, the groups were pooled. These tissues represent a mid-point in the metaplasia-dysplasia-carcinoma sequence, and could be combined together given their possible malignant potential; or separated with HGD more in keeping with adenocarcinoma, and assuming that adjacent Barrett's has not become part of the dysplastic "field change". When combined together ($n=6$), significantly more LTB_4 was produced (124.3 +/-38.1 pg/mg) compared to squamous (36.5 +/-4.1 pg/mg, $p=0.016$), with a trend to increased levels compared to Barrett's (63.0 +/-14.8

pg/mg, p=0.062). This was not seen with cys-LT. When the Barrett's next to HGD/adenocarcinoma cases were combined with Barrett's (n=19), and the HGD combined with adenocarcinoma (n=13); the significantly increased concentration of both LTB_4 and cys-LT from adenocarcinoma & HGD over Barrett's & Barrett's next to HGD/adenocarcinoma (p=0.004, p=0.001 respectively) and squamous tissue (p=0.001, p=0.007 respectively) was seen as above.

Figure 4.5 LTB_4 levels generated after 60 min of stimulation with A23187 (2 uM) in a range of oesophageal tissue types, including squamous epithelium, Barrett's oesophagus with and without dysplasia, and adenocarcinoma, as determined by EIA (bars represent mean).

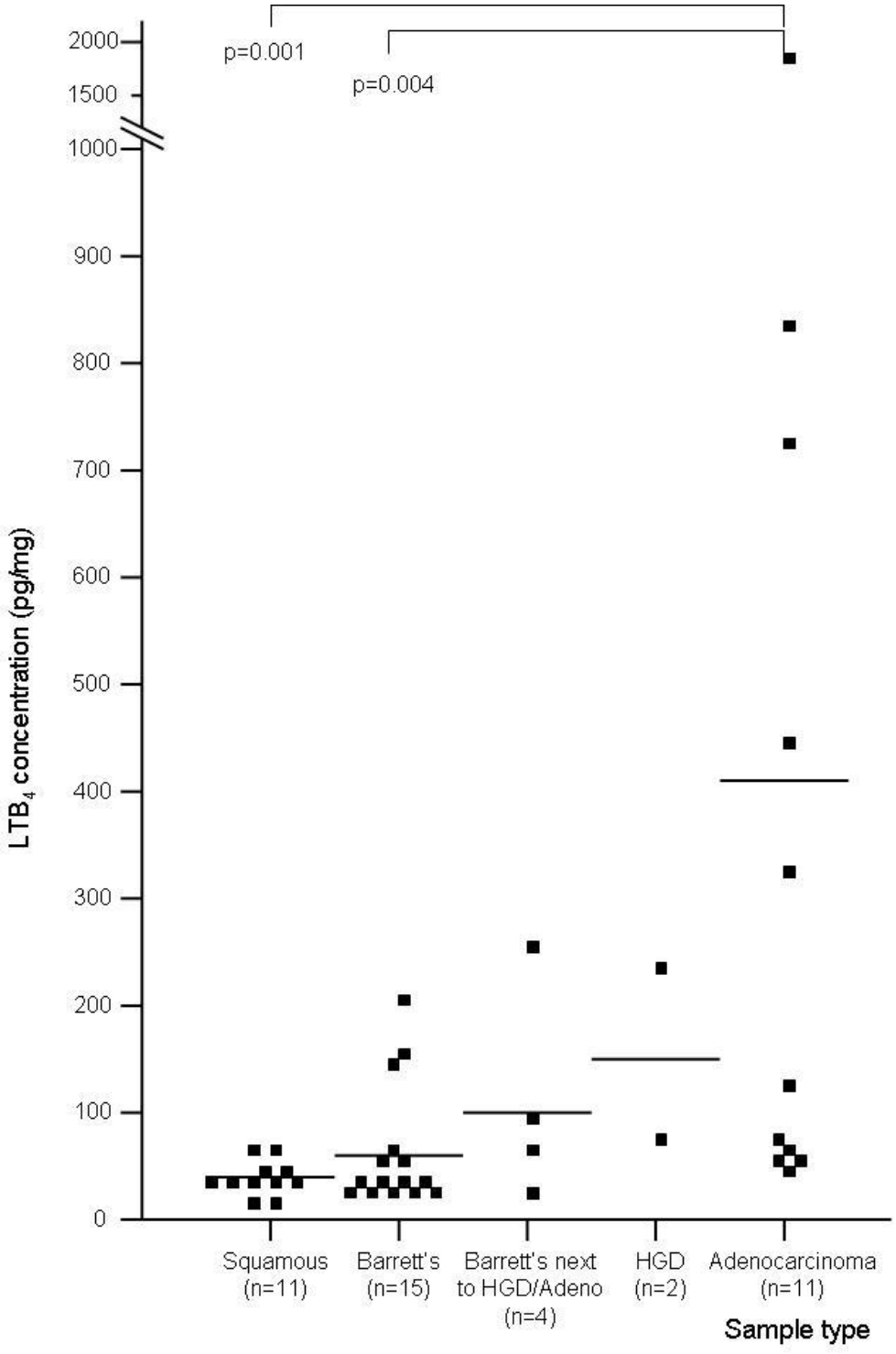
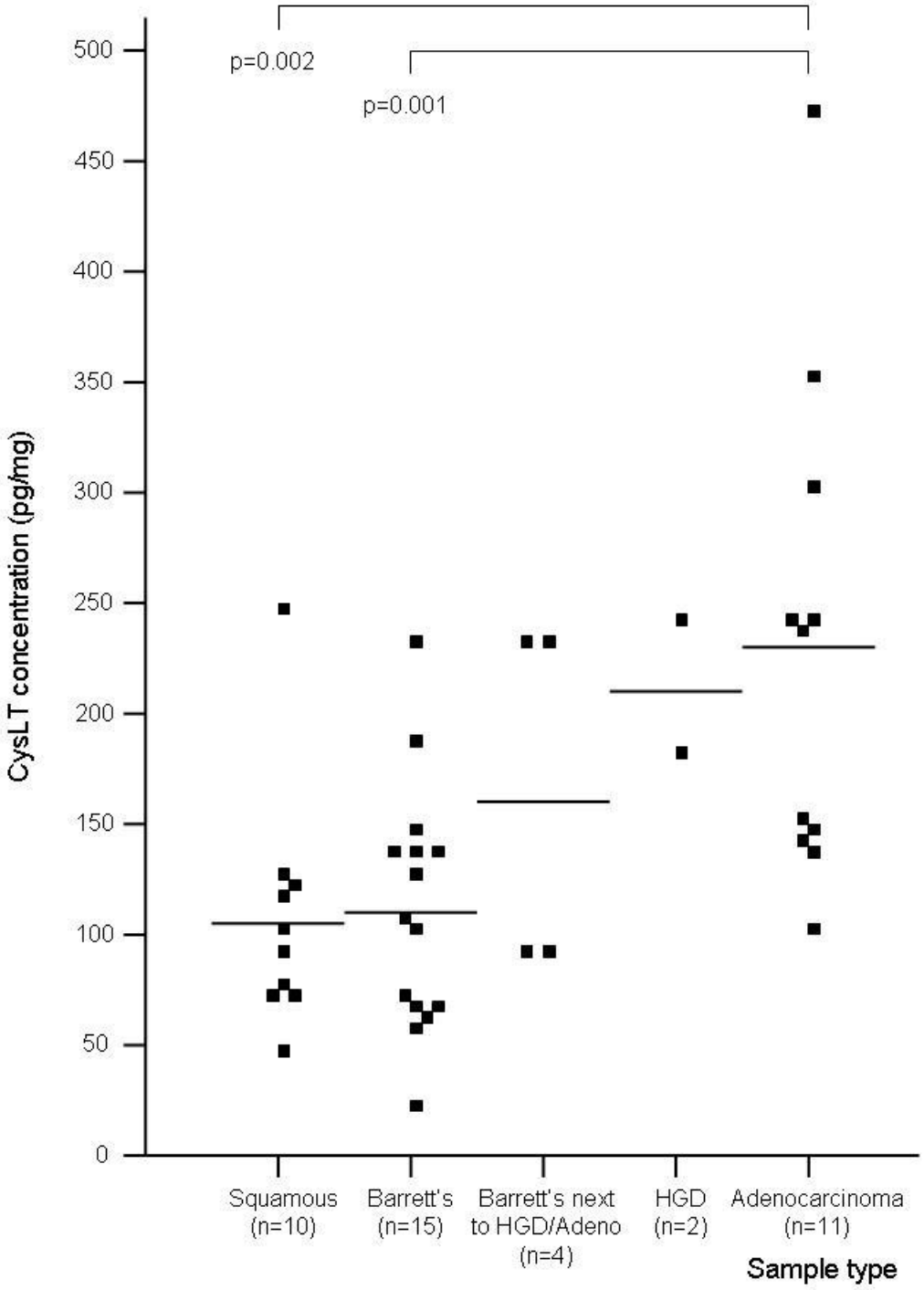


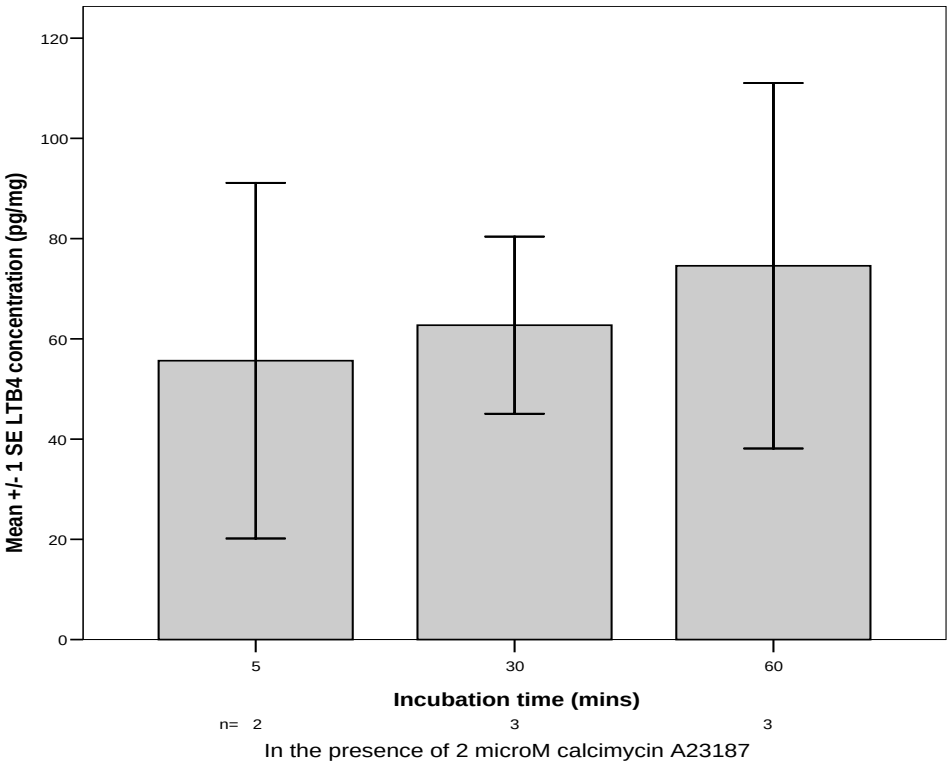
Figure 4.6 Cys-LT concentrations generated after 60 min of stimulation with A23187 (2 uM) in a range of oesophageal tissue types, including squamous epithelium, Barrett's oesophagus with and without dysplasia, and adenocarcinoma, as determined by EIA (bars represent mean).



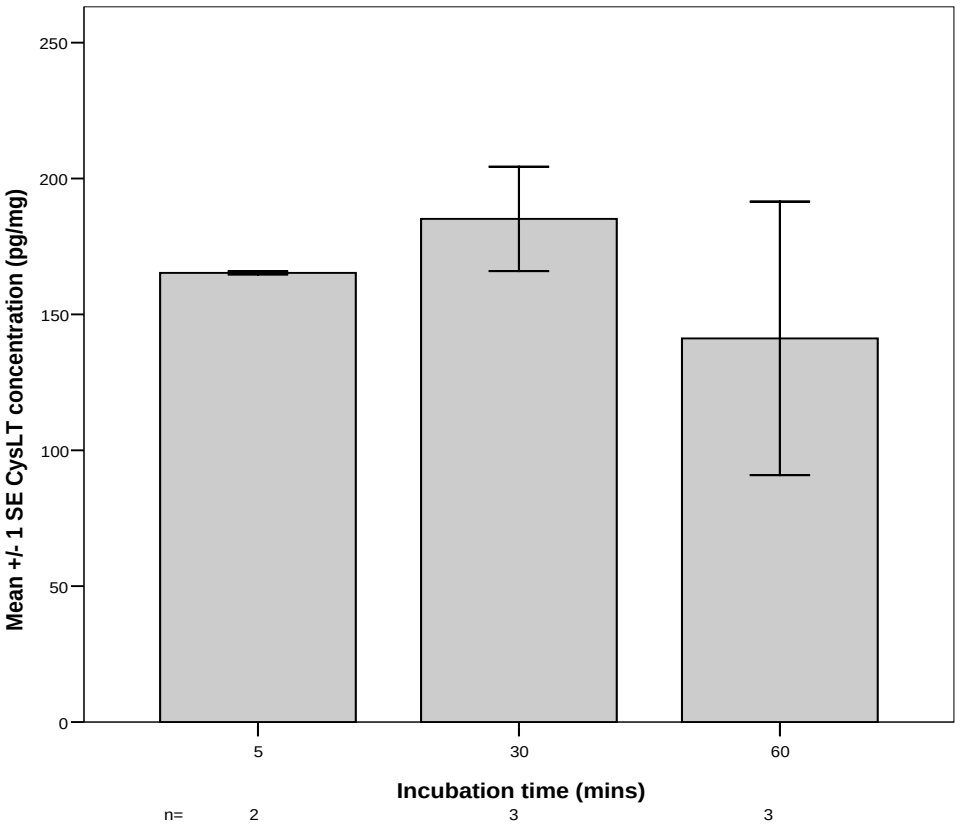
To begin to explore the time-course of leukotriene production and its dose-response relationships to A23187 stimulation, one tissue type (Barrett's oesophagus) was selected for further experiments. A preliminary time-course experiment was performed with three Barrett's oesophagus biopsy specimens being incubated in DMEM for 5, 30 and 60 minutes from the time of adding 2 uM calcimycin (A23187), following a 5 min pre-incubation to allow tissue to equilibrate.

There was a numerical trend towards increased LTB_4 concentration over time that was not seen with cys-LT, although no statistically significant differences were apparent across the time periods (Figure 4.7). Maximal levels of LTB_4 of 74.6 (+/- 36.5) pg/mg were achieved after 60 min, with mean cys-LT levels peaking at 185 (+/- 19.2) pg/mg after 30min.

Figure 4.7 LTB_4 and cys-LT concentrations determined by EIA produced by Barrett's oesophagus biopsies stimulated with A23187 (2uM) after 5, 30 and 60 min incubation.



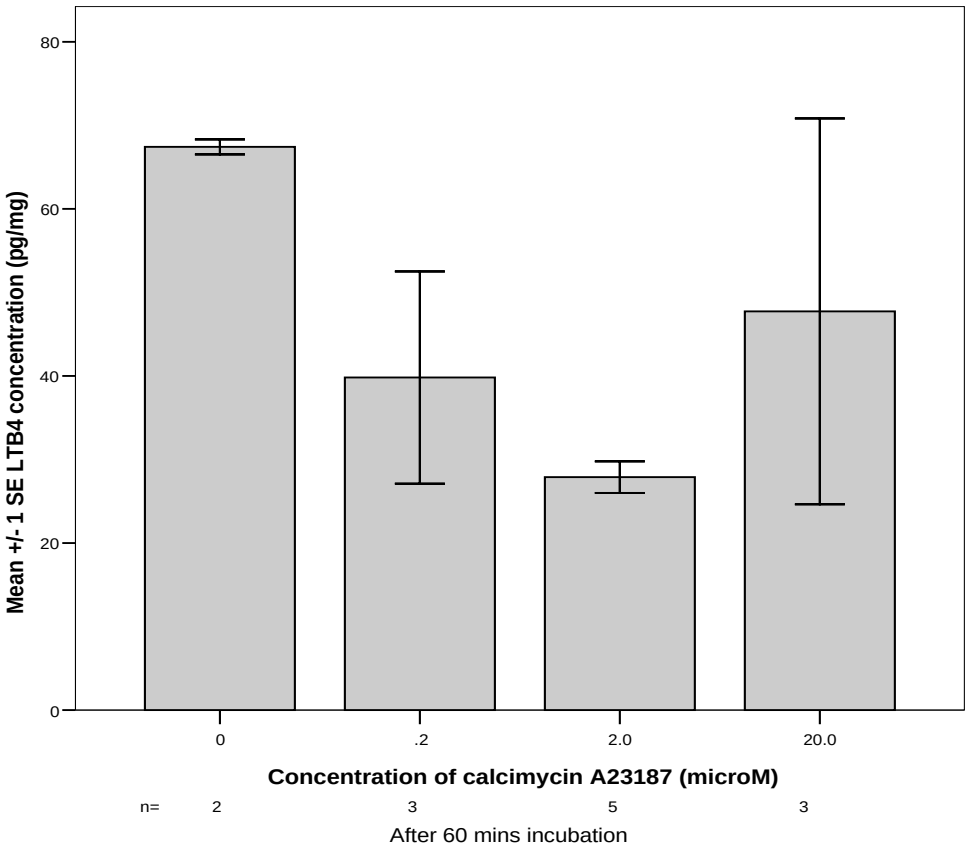
In the presence of 2 microM calcimycin A23187

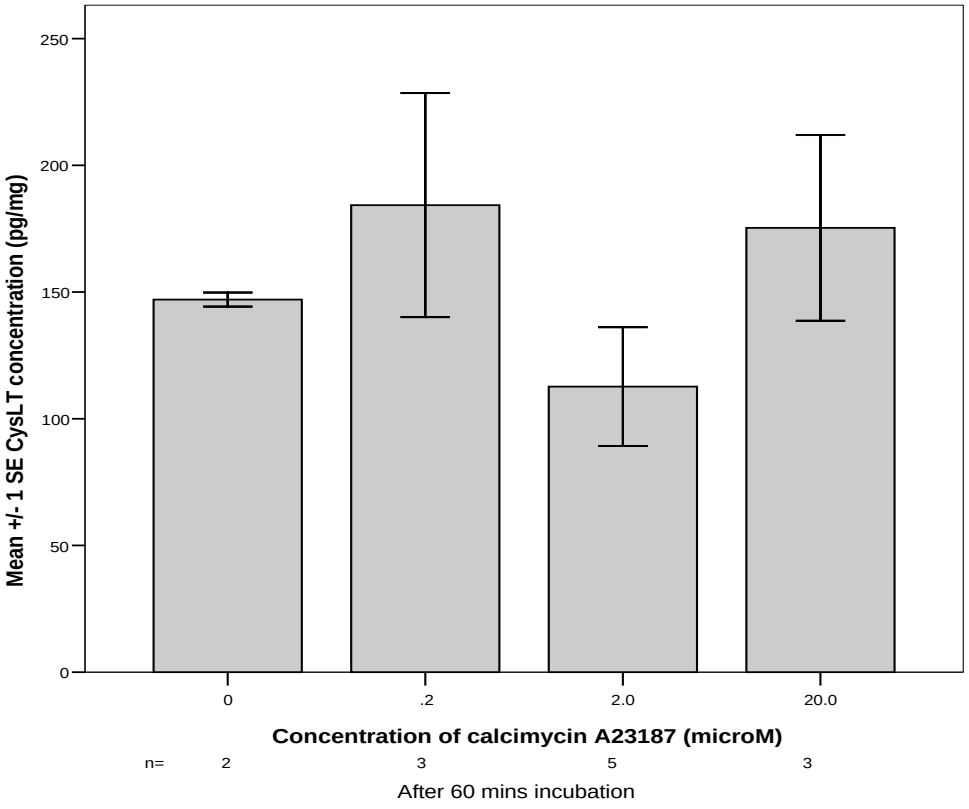


In the presence of 2 microM calcimycin A23187

To investigate the dose-response to increasing amounts of the ionophore calcimycin A23187, a serial dilution was prepared as described in Chapter 2.2.1. Calcimycin (A23187) was added to biopsy specimens of Barrett's oesophagus in final concentrations of 0, 0.2, 2 and 20 uM and incubated for 60 minutes. Unexpectedly, there was no convincing effect of the ionophore, with no statistically significant differences between concentrations, including zero (Figure 4.8).

Figure 4.8 LTB_4 and cys-LT concentrations at time 60 min after stimulation of Barrett's oesophagus biopsies (n=2-5) with a serial dilution of A23187 (0-20 uM).





This prompted further study of the handling of the biopsies to determine whether substantial leukotriene production is occurring during processing and/or during equilibration before the addition of the A23187 stimulus. Also, to confirm that the leukotriene immunoreactivity detected by EIA is authentic, the potent and selective FLAP inhibitor MK886 (quiflapon) was added to the culture medium of some biopsies to suppress leukotriene synthesis. The protocol and results are shown in Figure 4.9. The conditions were as follows:

(A) Biopsies placed in DMEM and weighed, before quenching with ice-cold methanol.

(B) As above but with a subsequent 5 minutes of incubation at 37°C before quenching with ice-cold methanol.

(C) Following weighing and equilibration, incubation for 10 min at 37°C with 10 uM MK886 alone, before methanol quenching.

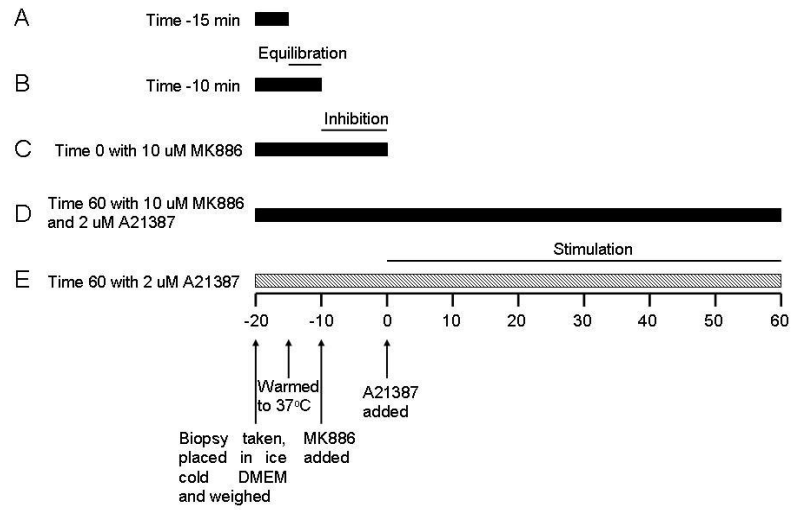
(D) Following weighing and equilibration, incubation for 10 min at 37°C with 10 uM MK886, then incubation for 60 min at 37°C with 2 uM A23187.

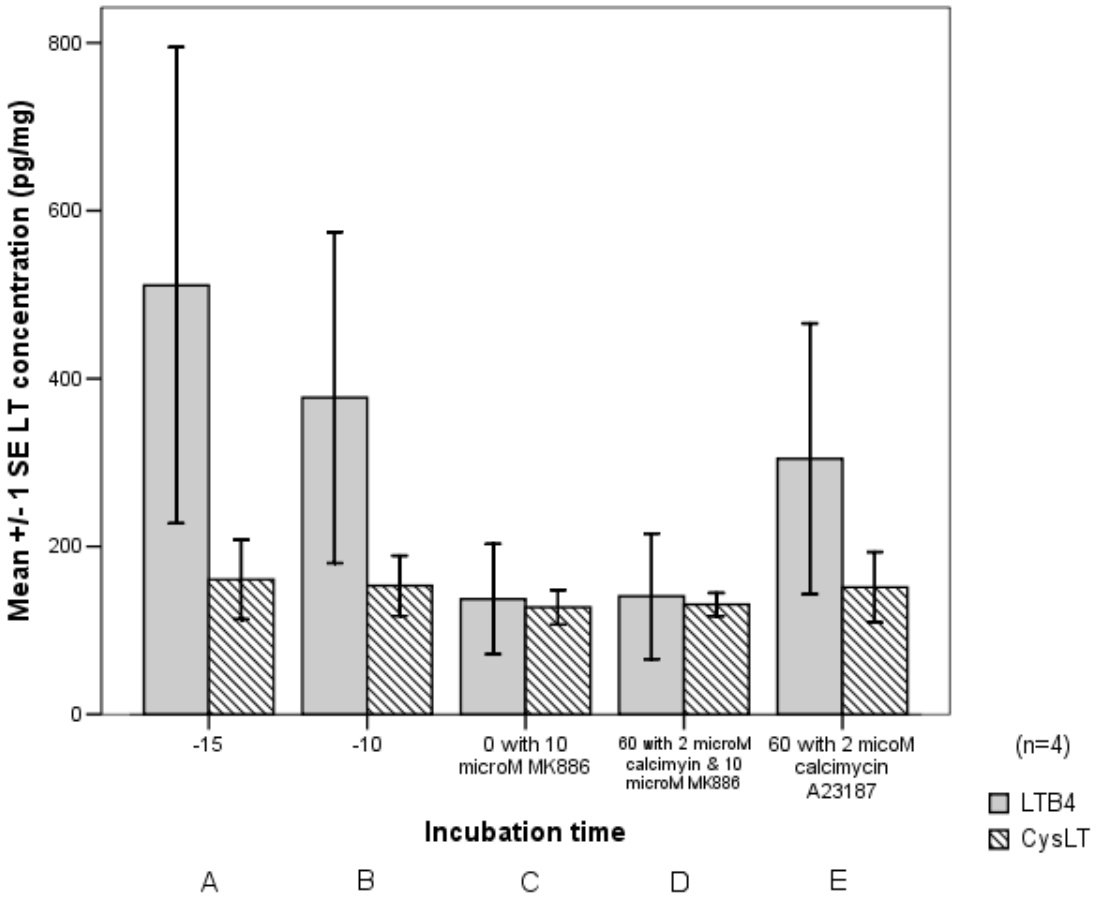
(E) As above, but without the 10 uM MK886 incubation for ten minutes.

These conditions test respectively whether leukotrienes are being generated during tissue weighing (condition A), during the 37°C pre-equilibration (B), during 37°C incubation with the FLAP inhibitor alone (C), or during incubations with the ionophore at 37°C for 1 hour in the presence (D) or absence (E) of the FLAP inhibitor. Each condition A-E was applied to 5 matched biopsies taken from 4 cases of Barrett's oesophagus (Figure 4.9).

The results of these experiments for these patients are presented in Figure 4.9 for LTB_4 and cys-LTs.

Figure 4.9 LTB_4 and cys-LT concentrations in four cases of Barrett's oesophagus from successive time points in the biopsy protocol as displayed in the timeline.





There were no significant or apparent differences in cys-LT concentrations between the five conditions A-E, with mean (SEM) concentrations immediately after weighing (condition A) of 161 (47) pg/mg not being affected by MK886 or A23187 treatments.

There appeared to be a numerically falling trend in LTB_4 concentrations from condition A (immediately after the biopsy was taken), amounting to mean (SEM) concentrations of 511 (283) pg/mg, falling to 137 (65.6) pg/mg after the 10-minute incubation with MK-886 (condition C). The presence of MK886 (condition E) also seemed to prevent the further increase in LTB_4 seen during the 60-min incubation with A23187 (condition D), but this did not reach statistical significance. These data may suggest production of leukotrienes occurring *in vivo* before biopsy together with a limited capacity of *in vitro* treatments (MK886, A23187) to penetrate the biopsy or modify its behaviour, and these aspects will be among those discussed below.

4.4 Discussion

The studies in this chapter set out to investigate the expression of 5-LO and FLAP by immunohistochemistry in a range of oesophageal tissues, from squamous mucosa, through the metaplasia-dysplasia sequence of Barrett's oesophagus through to adenocarcinoma. There was a significant increase in expression of 5-LO and FLAP in dysplastic Barrett's mucosa and adenocarcinoma compared to Barrett's. This was in keeping with the significantly increased expression of COX-2 in oesophageal adenocarcinoma compared to Barrett's, present in my work and previous studies (Shirvani et al. 2000;Bhandari et al. 2001). Furthermore, I aimed to quantify the end products of this enzyme cascade by measuring concentrations of LTB_4 and total cys-LTs in whole biopsy specimens from the same range of tissue sources, to investigate whether increased immunoexpression of the biosynthetic enzymes resulted in increased tissue generation of their biologically-active lipid products. Given the limited number and size of oesophageal biopsies and difficulties with combining laboratory methodologies to detect both proteins and lipids, an early decision was taken not to attempt to perform immunohistochemistry for the enzymes and immunoassays for the leukotrienes on the same samples. Sample collection and the subject groups were nevertheless comparable, enabling valid statistical comparisons to be made for each parameter between the disease groups and the squamous tissue controls. The results are believed to be the first to demonstrate significant upregulation ($p \leq 0.001$) of both 5-LO and its activating protein FLAP, together with significantly-increased increased generation of both the LTB_4 and cys-LT branches of the leukotriene family ($p \leq 0.002$), in human oesophageal carcinoma compared to Barrett's oesophagus or squamous biopsies. Unlike COX-2, both 5-LO ($p=0.005$) and FLAP ($p=0.004$) were also significantly increased in high-grade dysplasia (HGD) tissues when compared to squamous tissue, although a clear trend for this to be reflected also in enhanced LT generation may not have reached statistical significance due to low subject numbers in the HGD groups ($n=6$).

There are limited human data on the role played by leukotrienes in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence progression. A study by Triadafilopoulos *et al* (1996) measured LTB_4 and PGE_2 levels in human subjects with symptomatic gastro-oesophageal reflux disease (GORD) or Barrett's oesophagus and compared them to squamous tissue in asymptomatic controls with these findings (gastritis, duodenitis, gastric polyps, duodenal ulcer and normal tissue, in reducing frequency) (Triadafilopoulos et al. 1996). The mean LTB_4 levels for GORD (stages 2-4) (n=34) and Barrett's oesophagus (n=11) were both significantly higher than controls (n=70), at levels of 1212 pg/mg, 1339 and 665 pg/mg respectively, while PGE_2 levels showed no relationship to disease severity. The LTB_4 concentrations for GORD and Barrett's oesophagus appear to be a factor of 10 greater than our LTB_4 data, although differences in methodology make direct comparisons difficult. Measurements of LT generation reported in this thesis nevertheless incorporate significant advantages and improvements compared to the Triadafilopoulos study.

Firstly, Triadafilopoulos and colleagues measured total levels of each eicosanoid in oesophageal tissue that had been snap-frozen and homogenised before immunoassay (Triadafilopoulos et al. 1996). Their measurements would therefore represent the accumulated levels of LTB_4 and PGE_2 generated *in vivo* by intrinsic activity of the 5-LO and COX pathways within the tissue over an uncertain time-frame, plus any levels generated during the biopsy procedure and subsequent tissue handling before snap-freezing, minus any metabolism or degradation occurring in either state. Inhibitors of 5-LO (e.g. zileuton) or FLAP (e.g. MK886) could have been added immediately after sample collection to inhibit any LT synthesis *in vitro*.

In contrast, in the work reported here, live oesophageal tissue was resuspended in fresh culture medium and actively cultured at physiological temperature and pH to mimic *in vivo* behaviour. Eicosanoid synthesis over defined time-periods was stimulated with calcimycin (A23187), a pore-forming stimulus that bypasses receptor-dependent processes and directly activates both phospholipase A_2 and 5-LO by

selectively increasing intracellular Ca^{2+}. The LTB_4 and cys-LT concentrations measured after termination of the biosynthetic reaction with cold methanol thus represent the amounts of each leukotriene actively released via specific membrane carriers in relevant leukocytes into the culture medium. In addition, by subsequently incubating the cultures overnight in methanol at 4°C, the cytosolic leukotriene fraction was enabled to diffuse out of the leukocytes, to produce a total (extracellular and intracellular) estimate of LT synthesis.

It was further argued that the potency of calcimycin A23187 as a stimulus would ensure robust LT measurements reflecting the relative enzymatic activities of a highly-stimulated 5-LO pathway within different biopsies, and that the LT levels generated would be high enough to make any previous artefactual accumulation of LTs during the biopsy and tissue handling stages appear negligible by comparison. Although the experiments shown in Figures 4.7 to 4.9 suggest unexpectedly poor efficacy of the A23187 stimulus in this model compared to isolated cell suspensions, it can nevertheless be argued that this approach is a better model of the physiological activation of leukotriene-generating cells and more accurately reflects Triadafilopoulos' description of "*ex vivo* LT generation" than the assays of frozen, homogenised tissues applied in their study.

Secondly, in the Triadafilopoulos study (Triadafilopoulos et al. 1996), the snap-freezing and mechanical homogenisation of biopsy tissues was a much less gentle process than that used in the live tissue culture experiments reported in this thesis, and is likely to have caused significant release, solubilisation or emulsification of arachidonic acid (C20:4) and non-eicosanoid (C-22, C-18, C-16) fatty acids into the assay medium from disrupted and damaged cells. While the cross-reactivity to structural fatty acids of the anti-leukotriene antibodies used in most immunoassays may typically be <0.01%, the large quantities of such fatty acids present in frozen, then thawed and homogenised tissues may exceed the levels of potent receptor-active lipids (like the leukotrienes) by many orders of magnitude, perhaps up to 10^6-fold.

They therefore represent a significant potential source of non-specific immunoreactivity that could interfere with LT immunoassays, as documented in many studies of LT levels in tissues and biological fluids (Heavey et al. 1987;Westcott et al. 1990). In fact, they may account for the very high LTB_4 levels observed in the Triadafilopoulos study compared to the present work. While extensive prior purification is not required for accurate quantification of eicosanoids in cell or tissue culture supernatants, as in the present study, the use of a sample clean-up stage with solid-phase extraction (SPE) cartridges (e.g. C18 Sep-Pak), or a more extensive purification using high-performance liquid chromatography (HPLC), would have raised confidence in the authenticity of the LTB_4 immunoreactivity reported by Triadafilopoulos *et al*.

Thirdly, Triadafilopoulos and colleagues quantified LTB_4 and PGE_2 using radioimmunoassays (RIA) (Triadafilopoulos et al. 1996). Their paper gives no information on either the sensitivity or the specificity of their RIA, so it is not possible to compare these parameters with the more modern EIA kits used in the present work. However, RIA are generally less sensitive than enzyme immunoassays. The commercial EIA kits used in the experiments in this thesis had detection limits of 13 pg/ml for LTB_4 and 34 pg/ml for total cysteinyl-LTs, as well as having excellent specificity for their targets. The paper by Triadafilopoulos and colleagues (Triadafilopoulos et al. 1996) went on to show no meaningful relationships between their oesophageal levels of eicosanoids (LTB_4 or PGE_2) and symptom scores, conceivably because some of the eicosanoid levels may represent non-specific immunoreactivity. However, six weeks of oral treatment with the PPI omeprazole suppressed levels of the eicosanoids by 30-35% and improved both the histological appearance and patient symptom scores. The modest effect of PPI therapy on the eicosanoids probably reflects reduced acid irritation leading indirectly to reduced populations of LT-generating inflammatory cells.

The lab-based studies described here investigated not only the tissue-generated levels of each receptor-active leukotriene sub-family (LTB_4, cys-LTs), but also the tissue expression of both the proximal enzymes (5-LO and FLAP) that initiate the synthesis of leukotrienes. Both the enzyme expression and the lipid synthesis were strikingly related to Barrett's disease stage, with the highest levels being observed in adenocarcinoma. These data suggest systematic upregulation of both branches of the 5-LO pathway in Barrett's oesophagus and oesophageal adenocarcinoma. They add significantly to the literature which previously focused mainly on the 5-LO / LTB_4 axis, to the exclusion of the cysteinyl-LT branch, and by including patients with both Barrett's disease and oesophageal adenocarcinoma in the same study.

Chen and colleagues have nevertheless published a number of studies of arachidonic acid metabolism in human oesophageal disease, including those with adenocarcinoma, and also in the EGDA rat model of oesophageal adenocarcinoma, involving surgical anastomosis of the oesophagus to the duodenum (Hoque et al. 2005;Chen et al. 2002;Chen et al. 2004;Chen et al. 2003). In the EGDA model, increased levels of LTB_4 were detected using solvent extraction and liquid chromatography / mass-spectrometry on affected tissue homogenates, as well as higher levels of PGE_2 and of several hydroxyeicosatetraenoic acids (HETEs) derived from the 5-LO, 12-LO and 15-LO pathways, when compared to the unaffected proximal oesophagus (Chen et al. 2002). LTB_4 concentrations rose six-fold from 0.58 ng/mg of protein in the proximal oesophagus to 3.56 ng/mg in the inflamed oesophagoduodenal junction (the 5 mm proximal segment of oesophagus), and were 3-fold elevated in the adenocarcinoma samples (1.95 ng/mg). Corresponding levels of PGE_2 showed a similar pattern but were about 10-fold higher.

By analogy with experiments in this thesis using A23187 to liberate membrane-bound arachidonate, Chen and colleagues also supplied exogenous arachidonate to rat oesophageal homogenates. Although the concentration of arachidonate used is not reported, this resulted in further increases in LTB_4 levels, again with most being

produced at the oesophagoduodenal junction (6.37 ng/mg protein, after subtracting spontaneous LTB_4 production), followed by adenocarcinoma (2.89 ng/mg protein) and proximal tissue (0.66 ng/mg protein). In live rats receiving 40 weeks of oral treatment, non-specific inhibitors of COX (sulindac) and lipoxygenases (NDGA) had mixed effects in reducing PGE_2 and LTB_4 levels, as measured by EIA, and in preventing tumour incidence (Chen et al. 2002).

Chen and colleagues (Chen et al. 2004) later showed that 40 weeks of oral treatment with the specific 5-LO inhibitor zileuton or the COX-2-selective inhibitor celecoxib markedly reduced the histological incidence of rat oesophageal adenocarcinomas by around 50% and 70% respectively, although, curiously, tumour volumes were not affected, suggesting a role of both leukotrienes and prostanoids in tumorigenesis, if not in tumour growth. The combination of both drugs reduced tumour incidence by 91%, although the drugs suppressed oesophageal tissue levels of LTB_4 and PGE_2 respectively by only about 50%. In this case, levels of the eicosanoids in snap-frozen, homogenised tissues were detected using enzyme immunoassays after lipid extraction with organic solvent, and in the presence of exogenous indomethacin and zileuton to prevent *in vitro* eicosanoid synthesis.

Concentrations of LTB_4 in rat adenocarcinoma tissues are not easily comparable with the human data in this thesis, although in the two Chen studies (Chen et al. 2002;Chen et al. 2004) the LTB_4 levels in rat adenocarcinoma represent approximately 6-fold and 10-fold increases over background levels in normal rat oesophagus. This is similar to the approximately 10-fold increase in median LTB_4 levels generated in human oesophageal adenocarcinoma tissues in this thesis (Figure 4.5). Our study also showed increased levels of total cysteinyl-LTs generated in human adenocarcinoma, although compared to squamous tissue this amounted to just over a doubling of median levels (Figure 4.6). Nevertheless, this is an important and original result as the cysteinyl-LTs have pro-inflammatory activities additional to those of LTB_4, including microvascular

leak and contraction of vascular smooth muscle via CysLT1, CysLT2 and possibly other receptors.

The current study has also extended the literature by broadening investigations of the expression of the leukotriene biosynthetic pathway to include FLAP, the highly-inducible and rate-limiting accessory protein required for 5-LO activity in intact cells and tissues (Dixon et al. 1990). 5-LO expression has been demonstrated within epithelial tumour cells and some stromal cells in a majority of oesophageal adenocarcinomas (37 out of 53) (Hoque et al. 2005). In a series of 67 patients, Chen and colleagues used immunohistochemistry to report 5-LO expression at relatively high intensities in approximately 95% of human oesophageal adenocarcinomas, compared to only 23% of normal oesophageal tissues (n=22) (Chen et al. 2004). Small numbers of Barrett's disease (n=7) and dysplasia (n=9) samples showed intermediate levels of 5-LO expression. Chen and colleagues used a semi-quantitative scoring system to report 5-LO expression, whereas in the present report a more fully quantitative approach has been used to present 5-LO, FLAP and COX-2 immunostaining data, taking account of both the numbers of immunopositive cells and the relative intensity of immunopositive staining within them. Figures 4.1-4.3 clearly demonstrate highly-significant upregulation of both 5-LO and FLAP in both dysplasia and adenocarcinoma compared to squamous tissue, with COX-2 upregulated significantly only in adenocarcinoma.

Chen and colleagues separately studied LTA_4 hydrolase, the terminal enzyme for LTB_4 synthesis, by immunohistochemistry in human oesophagus (Chen et al. 2003). LTA_4 hydrolase expression was elevated in tissues from Barrett's (n=5), dysplasia (n=1), and oesophageal adenocarcinoma (n=86) patients, when compared to the parabasal cells of squamous oesophageal tissue (Chen et al. 2003). There was no statistical difference in staining intensity between dysplasia and adenocarcinoma. In the same study, when administered intra-peritoneally for seven days to rats with experimental oesophageal adenocarcinoma, bestatin, a poorly-selective drug that inhibits LTA_4 hydrolase and

some other aminopeptidases, reduced LTB_4 levels in the lower oesophagus by nearly 60% (Chen et al. 2003). In our experience, immunostaining for LTA_4 hydrolase using polyclonal antibodies tends to produce somewhat unsatisfactory results, with relatively poor specificity resulting in most epithelial cells staining uniformly positive and also generating high background staining in stromal cells in a variety of tissue types (unpublished data). In contrast, in view of the findings of elevated levels of total cysteinyl-LTs in human oesophageal adenocarcinoma (Figure 4.6), with intermediate levels in dysplasia, it would have been interesting to have explored oesophageal expression of LTC_4 synthase, the terminal enzyme for the synthesis of LTC_4, but at present antibodies for this key enzyme are unavailable.

Further light on the role of leukotrienes in oesophageal tumorigenesis is cast by experiments in cultured tumour cell lines by Hoque and colleagues (Hoque et al. 2005). The oesophageal adenocarcinoma cell lines SKGT-4 and SKGT-5, the Barrett's oesophagus cell line BE3, and five oesophageal squamous cell carcinoma (SCC) cell lines all expressed varying amounts of 5-LO, as detected by Western blotting. In the two adenocarcinoma cell lines, both LTB_4 production and cell viability were reduced after incubation with the first-generation 5-LO inhibitors REV-5901 and AA-861, accompanied by an increase in apoptosis quantified by flow cytometry. This indicates a key functional role of 5-LO products in sustaining survival of these tumor cells (Hoque et al. 2005). Indeed, exogenous LTB_4 was able to fully overcome the effect of the 5-LO inhibitors and restore low rates of apoptosis. These data suggest that *in vivo*, LTB_4 may be generated directly by adenocarcinoma cells, not just by infiltrating inflammatory leukocytes such as neutrophils, in amounts adequate to promote their own survival by reducing apoptosis, although LTB_4 from either source may contribute.

Curiously, Hoque and colleagues (Hoque et al. 2005) appear to have missed a key implication of one of their findings, which was that adequate concentrations of U-75302, a well-characterised and specific antagonist of the BLT1 receptor, induced apoptosis in only 6-13% of the SKGT-4 and SKGT-5 adenocarcinoma cells, compared

to 60-80% apoptosis induced by the 5-LO inhibitors (REV-5901 and AA-861). The first conclusion that could have been drawn was that endogenous LTB_4 was not inducing tumour cell survival by acting at its principal, high-affinity receptor (BLT1) (Yokomizo et al. 1997), but by another mechanism, possibly via its lower-affinity but ubiquitously-expressed BLT2 receptors (Yokomizo et al. 2000), or by acting at nuclear peroxisome proliferator-activated receptors (PPAR) (Narala et al. 2010). The effects of another BLT1 receptor antagonist, LY293111, in pancreatic cancer have been ascribed partly to its direct interaction with PPAR-gamma, independently of BLT1 blockade (Adrian et al. 2008). Such an unexpected interaction may also account for the paradoxical finding by Hoque and colleagues that U-75302 seemed to mimic, rather than oppose, the survival-enhancing activity of LTB_4 itself, a result that is otherwise hard to explain. A contribution to adenocarcinoma cell survival of other 5-LO products, such as cysteinyl-LTs acting at CysLT1 or CysLT2 receptors, was also not definitively excluded in the study by Hoque and colleagues (Hoque et al. 2005).

Overall, in the present study, the concentrations of LTB_4 and total cys-LTs generated in Barrett's disease tissue equated to about 15 nM and 4 nM respectively, which are well within the biologically-active ranges for these mediators, including the low nanomolar range of chemotactic activity of LTB_4 for human neutrophils and the similar potency of LTC_4/LTD_4 in increasing microvascular permeability (Ford-Hutchinson et al. 1980;Dahlen et al. 1981). The detected LTB_4 concentrations exceed those released by SKGT-4/5 oesophageal adenocarcinoma cell lines and reported to have profound suppressive effects on apoptosis in these tumour cells. The nanomolar concentrations reported here in human oesophageal tissues also exceed by 1-2 orders of magnitude the dissociation constants (Kd) for these agonists at their respective primary receptors, reported as a Kd of 0.154nM for LTB_4 at BLT1 receptors, and a Kd of 0.4nM for LTD4 at $CysLT_1$ receptors (Yokomizo et al. 1997;Lynch et al. 1999a). Again, this indicates that the detected concentrations should significantly activate these receptors, with likely activation of the lower-affinity BLT2 and CysLT2 receptors also. There are no previous data regarding the concentration of cys-LTs generated in human oesophageal tissue

specimens. Taken together, this study has demonstrated that significant concentrations of each leukotriene are released over short incubations (60 min) of whole endoscopic biopsy specimens, that they are readily detectable by EIA, and that the pattern of their release in the different oesophageal disease groups closely matches the differential expression of 5-LO and FLAP.

An area of difficulty in the present study nevertheless arose in interpreting the time-course and dose-response relationships between A23187 stimulation and leukotriene production in Barrett's oesophagus tissues, as shown in Figures 4.7 and 4.8. While relatively large numbers of biopsies were collected overall for this study, the limited amount of tissue collectable from individual subjects precluded extensive experimentation to examine dose-response and time-course relationships, because it was not possible to sub-divide individual biopsies to examine repeated time or dose conditions within the same tissue. While it was recognised in advance that access of even a small molecule like A23187 (molecular weight 524) to the interior of oesophageal biopsy specimens would be restricted, the relative lack of tissue response to this stimulus was unexpected. In the case of isolated PMN leukocytes cultured in suspension, A23187 stimulation at similar concentrations would be expected to increase LT production over a similar time-course in our experiments from approximately <500 pg per million cells to around 30000 pg/10^6 cells (Sampson et al. 1992). The flat responses of the oesophageal specimens, both over a range of times up to 60 min and a range of A23187 concentrations up to 20uM, suggest that the A23187 was not penetrating the biopsy specimen effectively, possibly due to a mucus layer masking some of the biopsy surface. Alternatively, the well-known LT hyperresponsiveness to activation by A23187 and other stimuli may be a characteristic of leukocytes not shared by epithelial or stromal cells, to the extent that LTs are sometimes mistakenly regarded as being generated only by myeloid cells. LT production is indeed relatively low in other cell-types such as epithelial cells and fibroblasts, even though expression of 5-LO and FLAP is comparable in these cells to that seen in blood leukocytes that generate much higher levels of LTs when stimulated

(James et al. 2007;James et al. 2006). LT generation in structural cell-types may be rate-limited by low expression of phospholipase A_2 and hence a low availability of arachidonate.

Another possibility to explain the lack of A23187 responsiveness is that the duration of stimulation was too short or the release of LTs from the biopsy specimens was too slow. Leukotriene synthesis is maximal within 15 minutes in leukocytes, and 5-LO then undergoes suicide inactivation, but epithelial cells and fibroblasts can sustain LT production at low levels for much longer (James et al. 2007;James et al. 2006). Our experiments should perhaps have included 12, 24 or 48-hour incubations to explore this. However, overnight incubation of tissues at 4^{o}C in an excess of cold methanol was intended to liberate any intracellular leukotrienes that had been synthesised, but not released from cells, and also to solubilise them effectively away from unwanted binding to structural lipids or other cell components.

Further timeline experiments to dissect the stage(s) during biopsy equilibration, pre-treatment and incubation at which the measured leukotriene concentrations were actually generated were hampered by the problem of biopsy size, as referred to previously. Nevertheless, in Figure 4.9, using freshly-obtained biopsies from a small number of different Barrett's subjects, LTB_4 and cys-LT levels were generally apparent and maximal at an early stage and varied little over the subsequent incubation. That this steady-state may nevertheless reflect a balance between active LT synthesis and ongoing degradation may be indicated by the modest effect of MK886, the FLAP inhibitor, which reduced LTB_4 levels at 60 minutes by perhaps 50%. .

It would seem therefore that the endoscopy, biopsy, and then the weighing and handling procedures before culture were sufficiently traumatic to generate substantial amounts of LTs in the biopsy specimens, and that further LT synthesis/release occurring during the equilibration and incubation periods may have been broadly matched by metabolism, degradation or other losses due to adhesion to tissues or

tube walls. To some extent, the outcome of our model may in fact more closely resemble the '*in situ* snapshot' of the LT biosynthetic capacity of oesophageal tissues achieved by the snap-freezing / homogenising method used by the group of Chen and by other workers than was originally intended, although our results are much less likely to have been confounded by non-specific immunoreactivity interfering with the immunoassays.

4.4.1 Future work

There are therefore a number of directions in which the current work could be pursued, both in the short-term to better validate and extend the range of techniques used to assess 5-LO pathway expression and activity in the disease and control groups, and in the mid- to long-term to identify more clearly the factors driving upregulation of this pathway, to define the cellular site of the upregulation (epithelial / stromal, infiltrating leukocyte, and/or adenocarcinoma cell), and to dissect the receptors and pathways by which leukotrienes may contribute to inflammation or tumorigenesis in the human oesophagus.

With regard to improving the current model of leukotriene synthesis, it is clear that as a highly potent and receptor-independent stimulus of intracellular calcium concentrations, calcimycin A23187 can be useful to estimate the maximal capacity of cells or tissue to generate leukotrienes in culture. This contrasts with approaches that estimate in frozen tissues the endogenous levels of leukotrienes that were generated spontaneously and present in the tissue *in vivo*. In the case of our oesophageal biopsies however, the poor access of the A23187 and/or the poor release of the resultant leukotrienes could be tackled by dispersing biopsy specimens, such as with collagenase, or by cutting the tissue into smaller pieces or slices to shorten the diffusion distances, using surgical scissors or a McIlwain tissue chopper, before washing and suspension in culture medium. It would also be appropriate to explore culture times up to 24 or 48 hours or longer, checking tissue viability at intervals with

lactate dehydrogenase (LDH) assays. Collection of tissue after culture would allow protein content to be estimated using the modified Lowry method, so that leukotriene production could be presented per milligram of cellular protein, as well as per gram of wet tissue weight. While the use of biopsy material takes advantage of relevant heterogeneous tissues containing physiologically accurate proportions of different cell-types, including the interactions between them, supporting the biopsy studies with experiments on defined cell-lines, including oesophageal epithelial cells and adenocarcinoma cells, would allow informative comparisons to be made. Well-characterised models of this type using human tissues could be used to test the *in vitro* actions and efficacy of 5-LO inhibitors, LT receptor antagonists, and perhaps dual 5-LO/COX inhibitors.

In this context, the unexpected lack of effect of the BLT1 antagonist U-75302 on the viability of oesophageal adenocarcinoma cell lines (Hoque et al. 2005) means that the identity and receptor activity of the 5-LO product(s) responsible for suppressing apoptosis in these cells is uncertain. While most oesophageal studies of leukotrienes have focused on the LTB_4 sub-family, the current study suggests parallel upregulation of the cys-LT pathway as well. While the currently available LTA_4 hydrolase antibodies have their limitations, and LTC_4 synthase antibodies are presently difficult to procure, in the past these have been used by our group and others to quantify expression of the respective pathways and localise them to specific cell-types in bronchial, dermal and gut biopsies (Cowburn et al. 1998;Jupp et al. 2007;Church et al. 2002). Depending on their availability, immunohistochemical analyses with these antibodies in oesophageal biopsies, and their co-localisation using double immunostaining or using the *camera lucida* technique on adjacent serial sections (Britten et al. 1993), could define the cell types and populations that contribute LTB_4 and cys-LTs in these tissues.

Immunohistochemistry could also be supplemented by quantification at the mRNA level. *In situ* hybridisation for each of the key enzymes is feasible, but studies are currently ongoing to use the Leica microdissection microscope to isolate specific cell

populations from oesophageal biopsies (as validated by Dr Philip Boger, Gastroenterology, Southampton University Hospitals) and to apply quantitative PCR assays for the key 5-LO pathway enzymes (developed by Dr John Holloway, III & Human Genetics Divisions, University of Southampton) to identify leukotriene cellular sources. A question of particular interest in both Barrett's disease and in adenocarcinoma is whether LT generation *in vivo* is from local infiltrating leukocytes and/or from structural oesophageal cells, whether or not transformed to a cancer phenotype. Evidence from oesophageal adenocarcinoma cell lines (Hoque et al. 2005) suggests that these contribute directly but this has not been quantified in the context of intact oesophageal tissue from patients with active disease. In view of the earlier discussion, it would be further worthwhile to extend the immunohistochemical analyses and the laser microdissection / PCR approach to include quantifying and localising the four key leukotriene receptors (BLT1, BLT2, CysLT1 and CysLT2) in the Barrett's disease, adenocarcinoma and control tissues. Antibodies for these receptors are available, some of them monoclonal, or polyclonal with appropriate control blocking peptides, and PCR assays are also being validated in our group for microdissection studies.

Oesophageal biopsies are also a useful model in which functional readouts relevant to Barrett's disease and adenocarcinoma could be studied, although these would benefit from longer-term study of tissue viability in culture. While such tissue explants lack a blood supply and therefore exclude the possibility of inward migration by leukocytes, nevertheless the effects of applying exogenous leukotrienes, 5-LO/FLAP inhibitors or specific LT receptor antagonists could be studied on markers of cellular activation, division/proliferation and apoptosis, such as the TUNEL microscopic technique. In parallel with studies in cultured cell lines, oesophageal tissue explants would provide additional information on cell-cell interactions including potential routes of transcellular synthesis of leukotrienes, as reported in the literature in other contexts (Zarini et al. 2009).

The use of oesophageal biopsies as tissue explants could also be developed for '*in vitro* interventional studies' to explore the impacts of known or suspected risk factors for Barrett's disease and oesophageal adenocarcinoma at the cellular level in intact tissue. The readouts would include the methods above for assessing the expression and/or activity of the 5-LO pathway and LT receptors at the mRNA, protein and lipid levels, and their functional effects on cell division and apoptosis. These *in vitro* studies could include treatment with:

(i) Classical (non-selective) NSAIDs such as indomethacin.

(ii) Low pH media (using HCl) to mimic exposure to gastric acid.

(iii) Graded solutions of ethanol.

(iv) Solubilised tobacco smoke.

Time-course and dose-response relationships would be defined for the effects of these stimuli on expression of 5-LO and COX pathway enzymes and receptors (using laser microdissection/PCR assays and immunohistochemistry), and the bioactive lipids (using LT and PG enzyme immunoassays). The modulatory effects of the stimuli on both eicosanoid families could also be applied to fragmented / sectioned tissues treated with Ca^{2+}-dependent triggers such as A23187, anti-IgE (targeting mast cells), or lipopolysaccaride (targeting mononuclear cells). Novel families of anti-inflammatory lipids such as the lipoxins, resolvins, protectins and maresins are increasingly coming to the forefront of inflammation and cancer research (Serhan et al. 2008;Serhan et al. 2009). The generation and release of these agents from oesophageal tissues in different disease states would present novel avenues of exploration, using immunoassays combined with mass spectrometry, to determine the factors that underlie vulnerability to Barrett's oesophagus and adenocarcinoma.

5 OVEREXPRESSION AND GENE AMPLIFICATION OF TOPOISOMERASE II ALPHA IN OESOPHAGEAL ADENOCARCINOMA

5.1 Introduction

Patients with oesophageal adenocarcinoma often present with advanced disease and with a poor prognosis despite modern treatment regimes, with many cases inoperable at diagnosis. The search for biomarkers to aid clinical management has included the *HER-2/neu* (*HER-2*) oncogene located on chromosome 17 and its product HER-2. Over-expression of HER-2 amplification of *HER-2* using FDA-approved techniques in invasive breast cancer is associated with poor survival and is used to identify patients who may respond to treatment with the humanized monoclonal antibody trastuzumab (Herceptin®, Roche pharmaceuticals) (Ross & Fletcher 1999). The majority of studies of HER-2 over-expression in tumours of the gastrointestinal tract indicate a potential prognostic value, with the exciting potential for new therapeutic approaches (Ross & McKenna 2001;Tanner et al. 2005).

In a previous study by our group, we investigated HER-2 protein over-expression and gene amplification in archival tissue from 100 resected oesophageal adenocarcinomas, using immunohistochemistry (Dako Herceptest®) and fluorescence *in situ* hybridization (Vysis PathVysion®) respectively. A minimum of five years clinical follow up was available. The relationship of protein over-expression and gene amplification to histopathological tumour characteristics and survival was determined. We concluded that HER-2 over-expression (IHC2+/FISH+ or IHC3+) and *HER-2* gene amplification are identifiable in 5% of oesophageal adenocarcinomas (HER-2 'positive') using FDA-approved methods for predicting trastuzumab response in breast cancer. It is possible that these HER-2 'positive' poor prognosis tumours may also respond to trastuzumab therapy. There was a significant correlation between protein over-expression and amplification ($p<0.01$), although two cases of chromosome 17 polyploidy were found to exhibit HER-2 IHC scores of 2+ and 3+. However, a significantly higher risk of death was only seen when cases with *HER-2* gene amplification or chromosome 17 polyploidy were combined, suggesting the presence of other oncogenic mechanisms underlying the more aggressive behaviour of these two subgroups. An in vitro study of

a HER-2 expressing oesophageal adenocarcinoma cell line showed that trastuzumab with radiotherapy could induce cell death, and a phase I/II study of trastuzumab with cisplatin and paclitaxel in oesophageal adenocarcinomas with strong HER-2 expression pointed to increased survival, suggesting a possible role for HER-2 inhibition (Sato et al. 2005;Safran et al. 2007).

Previous immunohistochemical studies have demonstrated marked variation in the presence of HER-2 over-expression in oesophageal adenocarcinoma (11% to 73%) (Jankowski et al. 1992;Flejou et al. 1994;Nakamura et al. 1994;Hardwick et al. 1995;Hardwick et al. 1997;Polkowski et al. 1999), with a similar variation in *HER-2* gene amplification using dual colour fluorescence *in situ* hybridization (FISH) (32% to 83.3%) (Walch et al. 2004;Walch et al. 2001;Rossi et al. 2010;Walch et al. 2000a). Chromosomal aneuploidy is a frequent early event in Barrett's-associated carcinogenesis, occurring in 60-100% of adenocarcinomas (Beuzen et al. 2000;Ferrando et al. 1998). Intense HER-2 expression has been described in oesophageal adenocarcinomas with gene amplification, while moderate and weak protein expression has been identified in tumours with chromosome 17 polysomy alone and in other tumours showing no specific oncogene amplification (Walch et al. 2001;Geddert et al. 2002). This has been described in cancers of the breast, ovary, salivary gland and endometrium (Jimenez et al. 2000;Underwood et al. 1995;Press et al. 1994;Saffari et al. 1995). Therefore there may be regulation of protein expression at the transcriptional or post-transcriptional levels, as well as a consequence of oncogene amplification (Geddert et al. 2002). The prognostic significance of HER-2 over-expression and *HER-2* gene amplification in oesophageal adenocarcinoma remains uncertain (Ross & McKenna 2001).

Topoisomerase II alpha (Topo IIα) is a key enzyme in DNA replication, excising and reconnecting double-stranded super-coiled DNA during the replicative cell cycle, and is a target of many chemotherapeutic agents such as the anthracyclines epirubicin and doxorubicin (Froelich-Ammon & Osheroff 1995;Jarvinen et al. 2000). The gene lies

close to *HER-2* on chromosome 17 (Smith et al. 1993). Breast cancers that over-express HER-2 are frequently responsive to anthracycline-based chemotherapeutic regimens (Paik et al. 2000). When *HER-2* is amplified in breast cancer, many cases also show *topoisomerase II alpha* (*Topo IIα*) gene amplification or deletion (Tanner et al. 2001) (Jarvinen et al. 1999;Bouchalova et al. 2006), with an association with adverse tumour characteristics and decreased survival in those with amplification or over-expression (Depowski et al. 2000;Callagy et al. 2005). Further studies revealed that amplification of *Topo IIα* may predict response to anthracycline-based therapies, with improved survival (Coon et al. 2002;Jarvinen et al. 2000;Di Leo et al. 2002;Kawachi et al. 2010). However some studies suggest that *Topo IIα* gene amplification does not result in over-expression of Topo IIα protein (Mueller et al. 2004), and whether another mechanism than gene amplification is responsible for the increased responsiveness to doxorubicin-based chemotherapeutic regimens (Harris et al. 2009). A recent study suggests that the Topo IIα protein expression and not gene amplification predicts responsiveness to adjuvant anthracycline-based chemotherapy in primary breast cancer (Schindlbeck et al. 2010). Amplification of both genes has also been reported in carcinomas of the prostate, stomach, colon and pancreas (Murphy et al. 2007;Hansel et al. 2005;Kanta et al. 2006;Al-Kuraya et al. 2006). Rossi *et al* have recently published their study of Topo IIα and HER-2/neu over-expression / amplification in Barrett's oesophagus, dysplasia and adenocarcinoma (Rossi et al. 2010). They utilized immunohistochemistry and FISH in 18 cases of Barrett's, 13 cases of Barrett's with dysplasia (5 low grade dysplasia, 8 high grade dysplasia), and 13 cases of oesophageal adenocarcinoma. They found a significant association between Topo IIα over-expression and *Topo IIα* amplification, chromosome 17 aneuploidy, *HER-2* amplification, and HER-2 over-expression as well as between HER-2 over-expression and *HER-2* amplification, *Topo IIα* amplification, and chromosome 17 aneuploidy. Gene amplification (*HER-2* or *Topo IIα*), protein over-expression (HER-2 or Topo IIα), and chromosome 17 aneuploidy were associated with dysplasia or adenocarcinoma.

In the present study, I tested the hypothesis that *Topo IIα* amplification or deletion is a frequent genetic event in oesophageal adenocarcinoma in the same series of 100 archival specimens of resected oesophageal adenocarcinomas, and I correlated this with protein expression, *HER-2* amplification, histopathological tumour characteristics and clinical outcome.

5.2 Methods

5.2.1 Tissue Samples

Routine formalin-fixed, paraffin wax-embedded material that was surplus to diagnostic requirements from 100 consecutive oesophageal adenocarcinoma specimens, resected between 1991 and 1996, had been identified from the archives of the Cellular Pathology Department at Southampton General Hospital for the HER-2 study. Patients known to have received neoadjuvant therapy were excluded from the study. The haematoxylin and eosin stained slides and respective paraffin blocks had been retrieved for histopathological review by an experienced gastrointestinal pathologist (ACB), including pTNM classification according to the UICC criteria (2002).

In 8 cases the blocks chosen were unsuitable for further analysis, with no further blocks available. In 9 further cases alternate blocks from the tumour had to be chosen and were reviewed by the same experienced GI pathologist (ACB). Therefore there were a total of 92 specimens available for the present study. Ethical approval was granted by the Southampton Local Research Ethics Committee (06/MRE12/38).

5.2.2 Immunohistochemistry

Immunohistochemistry was performed as described in section 2.1 on 3 µm sections cut from the 92 oesophageal adenocarcinoma blocks available, using 200 µl primary mouse anti-human Topo IIα antibody (Novocastra, UK) at a 1:40 dilution. A positive control slide was also included in each run, consisting of a 3 um thick section of human tonsil treated in an identical manner. A negative control slide for each case consisted of an immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the primary antibody to Topo IIα.

5.2.3 Immunohistochemistry interpretation

The completed slides were scored for Topo IIα immunoreactivity by counting the proportion of all adenocarcinoma cells showing positive nuclear staining in 10 random high power fields (x400) using an eyepiece graticule (mean total cell count per high power field per case of 97, range 30-214). A mean labeling index for each case was then calculated and was expressed as a fraction of 1.00.

5.2.4 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed as described in section 2.1, initially on a slide bone marrow aspirate to confirm probe activity. To investigate ideal conditions for the protease and codenaturation steps, 3 um sections of human tonsil and surplus oesophageal adenocarcinoma tissue were analysed. Thereafter, 3 um sections cut from the 92 oesophageal adenocarcinoma blocks were assessed.

5.2.5 Statistical analysis

The Topo IIα immunohistochemistry and FISH findings were matched with anonymised (unlinked) clinical data to assess the correlation with *HER-2* amplification, histopathological tumour characteristics and clinical outcome using Pearson Chi squared and Fisher's exact test. Median survival times were calculated. Comparison of survival time was made by combining Kaplan-Meier and Log Rank analysis, with a Cox regression model. Cox proportional hazards assumptions were checked using log (analysis time) against -log (log [survival probability]). A value of $p<0.05$ was considered as statistically significant.

5.3 Results

5.3.1 Clinical characteristics

There were 84 men and 8 women, with an age range of 39 to 83 years (mean 66 years). With regards to stage, 3 (3%) were pT1, 11 (12%) pT2 and 78 (85%) pT3; 23 (25%) were stage pN0, 69 (75%) pN1 and 1 (1%) M1.

5.3.2 Topoisomerase IIα immunohistochemistry

The labeling indices for all 92 cases are displayed in Figure 5.1. There was a wide continuous variation in labeling index, with a mean of 0.23, median 0.2, and range 0-0.78. There was a trend to an increase in the mean labeling index with chromosome 17 polyploidy and *Topo IIα* amplification, but the differences were not statistically significant by Mann-Whitney U-test (Figure 5.2). With no obvious stepwise change in labeling index, an arbitrary cut off of 50% of maximal labeling index was chosen to separate those tumours above which immunohistochemical staining was deemed positive, and below which was negative. Using this criterion, Topo IIα protein over expression was identified in 18 cases (20%) (Table 5.1) and this correlated with the presence of *Topo IIα* gene amplification ($p=0.03$, Fisher's exact test) (Table 5.2), and when *Topo IIα* amplification was combined with chromosome 17 polyploidy ($p=0.02$, Chi squared test). Representative photomicrographs are shown in Figure 5.3. There was no correlation between Topo IIα over-expression and survival.

Figure 5.1 Topo IIα labeling index of 92 cases of oesophageal adenocarcinoma. The 50% relative labeling intensity cut off is shown, above which immunohistochemical staining was deemed positive, and below which was negative.

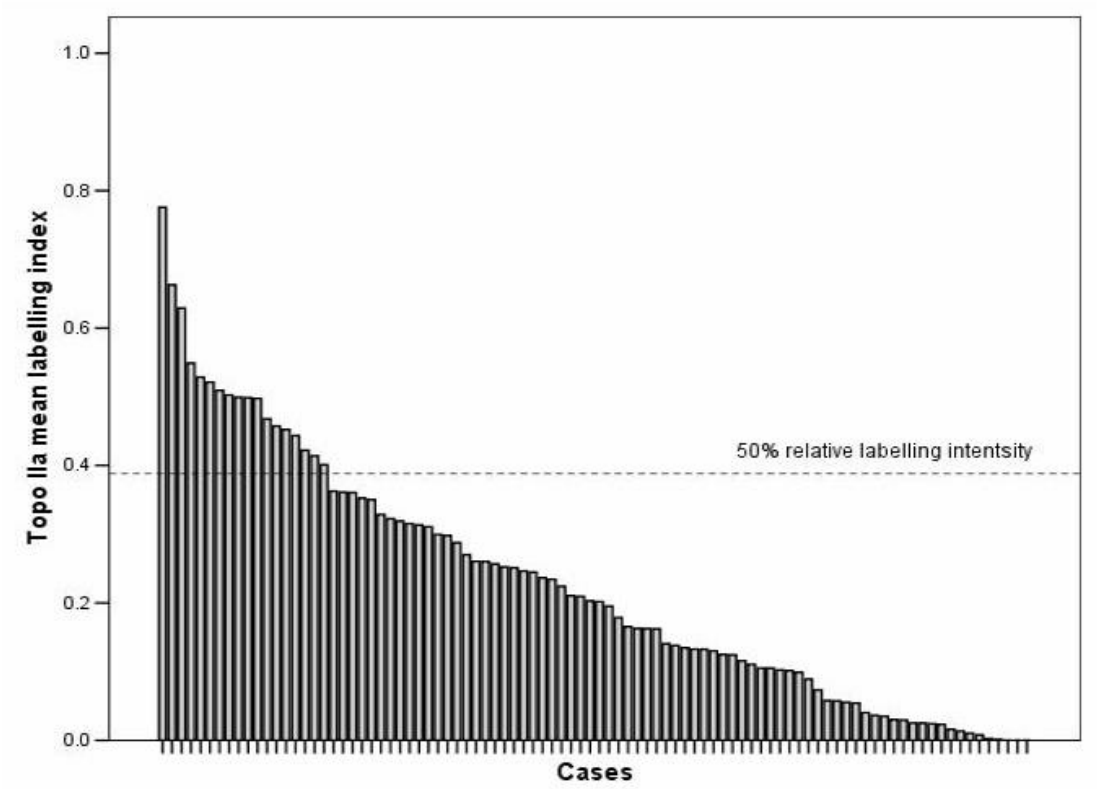


Figure 5.2 Mean labeling index +/- 1 standard error of the mean for Topo IIα immunohistochemistry by gene status: diploid or polyploid chromosome 17, *Topo IIα* deletion or amplification. The differences in means were not statistically significant by Mann-Whitney test.

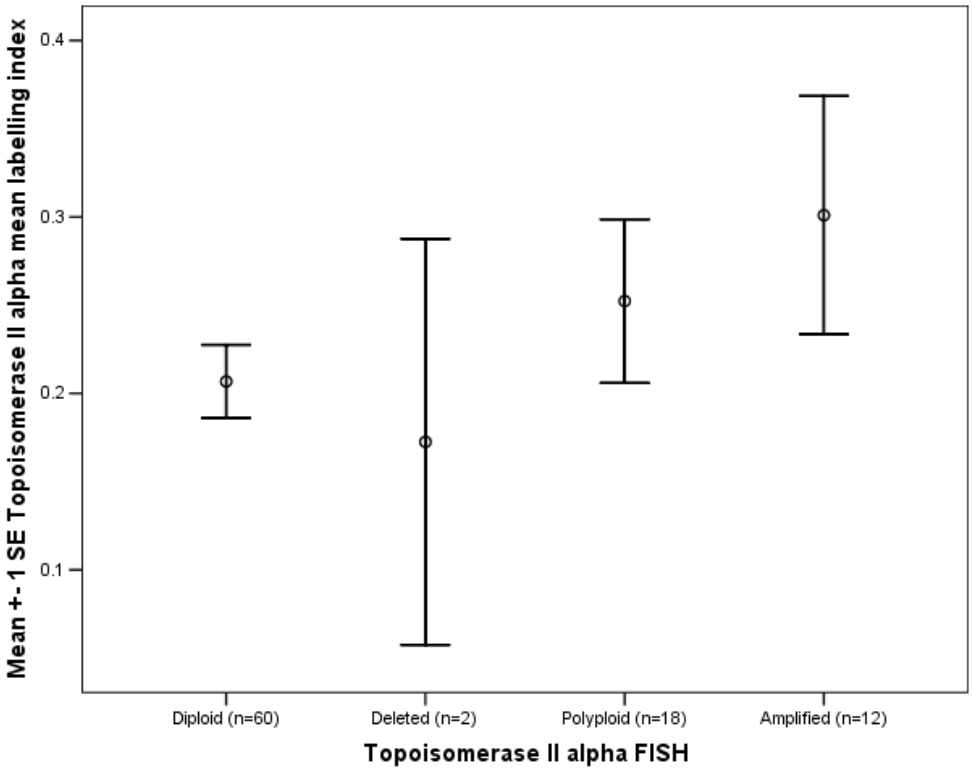
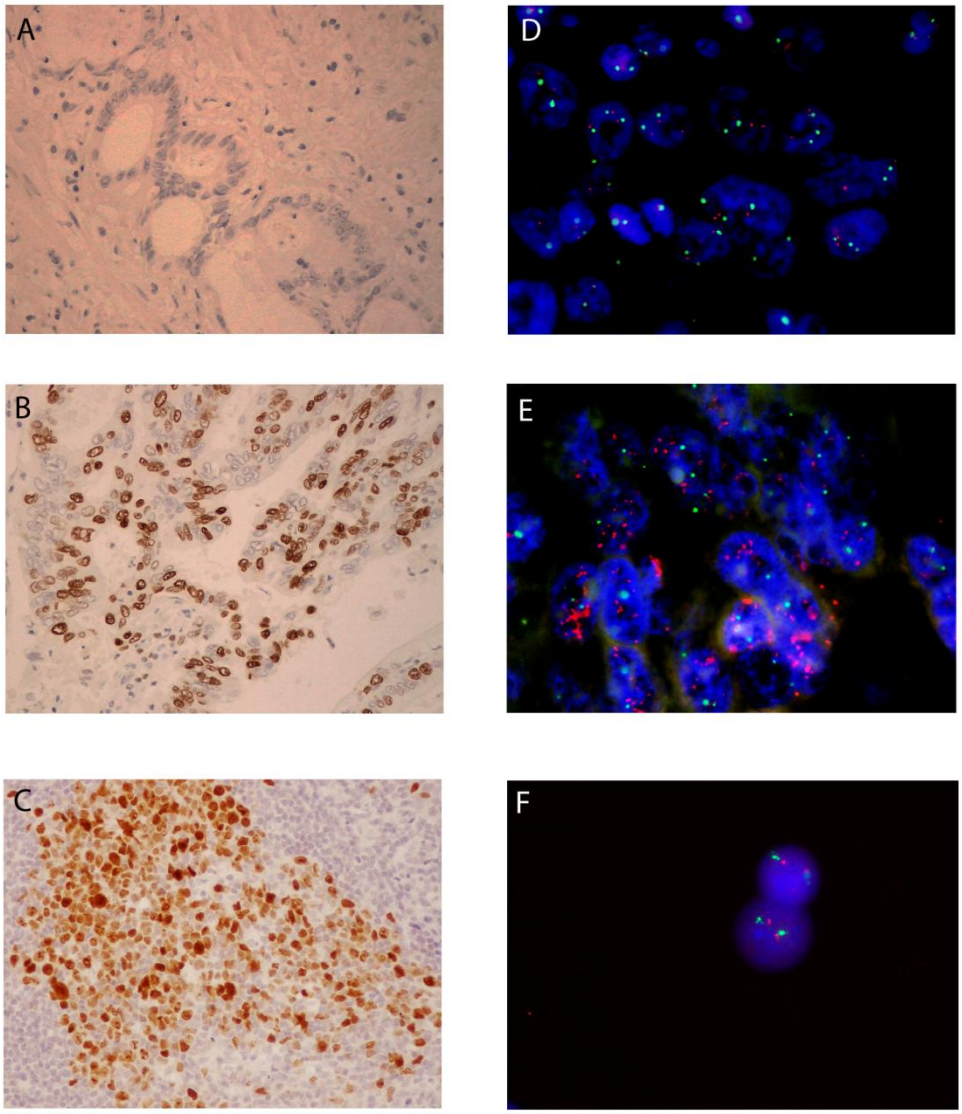


Figure 5.3 Light microscopy pictures of oesophageal adenocarcinoma prepared by immunohistochemistry for Topo IIα, with negative staining (A), and strong staining (B), with control tonsil tissue (C) (magnification x200). Dual colour FISH images of oesophageal adenocarcinoma with spectrum orange and spectrum green fluorescent DNA probes specific for the *Topo IIα* locus and chromosome 17 centromere respectively, showing,chromosome 17 trisomy (D) and *Topo IIα* amplification (E), with diploid chromosome 17 in a control bone marrow aspirate (F) (magnification x1000).



5.3.3 *Topoisomerase IIα* amplification

Two chromosome 17 centromere signals and two *Topo IIα* signals were clearly seen in a bone marrow aspirate (Figure 5.3). Tonsil sections prepared in an identical manner, with exposure to the protease for 10 min and codenaturation melt temperature of 85°C for 1 min, produced some specific labeling but a significant level of background staining. Tonsil sections were exposed to protease for 15 and 20 min, with minimal background staining apparent with the 20 min incubation. The chromosome 17 centromere signals were good, confirming the probe had entered the nucleus. However, the *Topo IIα* signals were less clear, suggesting inadequate denaturation of DNA. Further extension of the protease time to 25 min and a codenaturation melt temperature of 85°C for 1 and 2 min, and at 90°C for 1 min, failed to improve the quality of signals. A decreased melt temperature but for a longer duration was subsequently assessed, with tonsil sections exposed to protease for 10 or 20 min, then denatured at 72°C or 75°C for 5 min. Optimum probe entry into nuclei and hybridization with target DNA was obtained with exposure to protease for 20 min and denaturation at 75°C for 5 min.

The 92 cases of oesophageal adenocarcinoma were then analysed. Twelve (13%) showed *Topo IIα* amplification. Nine cases had a *Topo IIα*:centromere copy number ratio of 2, one case with 4, and two cases of 5. *Topo IIα* deletion was seen in 2 cases (2%), both in the setting of chromosome 17 trisomy. Chromosome 17 polysomy without *Topo IIα* amplification was observed in 18 cases (20%). The remaining 60 cases (65%) showed no evidence of *Topo IIα* amplification or deletion, or chromosome 17 polysomy (Table 5.1). Representative photomicrographs demonstrating oesophageal adenocarcinoma nuclei that are polyploid for chromosome 17, and those with *Topo IIα* amplification are displayed in Fig 5.3.

Table 5.1 Summary of findings of FISH for *Topo IIα*, and immunohistochemistry for Topo IIα in 92 cases of oesophageal adenocarcinoma.

Topoisomerase IIα expression	FISH for *Topoisomerase IIα*				Total
	Deleted	Diploid	Polyploid	Amplified	
Negative	2	52	13	7	74
Positive	0	8	5	5	18
Total	2	60	18	12	92

5.3.4 Clinicopathological associations

There was no significant association between *Topo IIα* gene amplification or deletion or chromosome 17 polysomy and sex, age at diagnosis, pathological tumour stage, presence of lymph node metastasis, or degree of tumour differentiation (Table 5.2). As mentioned above, *Topo IIα* amplification was significantly associated with Topo IIα protein expression, as well as with HER-2 protein expression and *HER-2* gene amplification ($p<0.01$, Fisher's exact test).

The overall survival of the 92 patients with resected oesophageal adenocarcinoma was a median of 14 months (range 0 to 146 months). The mean survival of patients with tumours showing *Topo IIα* gene deletion, amplification, chromosome 17 polysomy, or none of these features was 12, 19, 11.5 months and 15.5 months respectively. Cox regression analysis indicated that the presence of lymph node metastases and poor or undifferentiated tumour was strongly associated with impaired survival ($p<0.01$) (Table 5.3). There was no significantly increased risk of death with tumours with *Topo IIα* amplification or chromosome 17 polyploidy over tumours that were diploid for chromosome 17. Kaplan-Meier analysis demonstrated no significant difference in survival of cases with *Topo IIα* amplification or chromosome 17 polyploidy, compared to cases that were diploid for chromosome 17 without *Topo IIα* amplification (Figure 5.4A-B). There was no significant difference in survival when cases with *Topo IIα* amplification or chromosome 17 polyploidy were combined and compared to cases that were diploid for chromosome 17 without *Topo IIα* amplification (Figure 5.4C). There were insufficient cases with *Topo IIα* deletion to analyse survival.

Table 5.2 Clinical and histopathological associations of chromosome 17 diploid, polyploid, *Topo IIα* deletion and amplification in 92 cases of oesophageal adenocarcinoma.

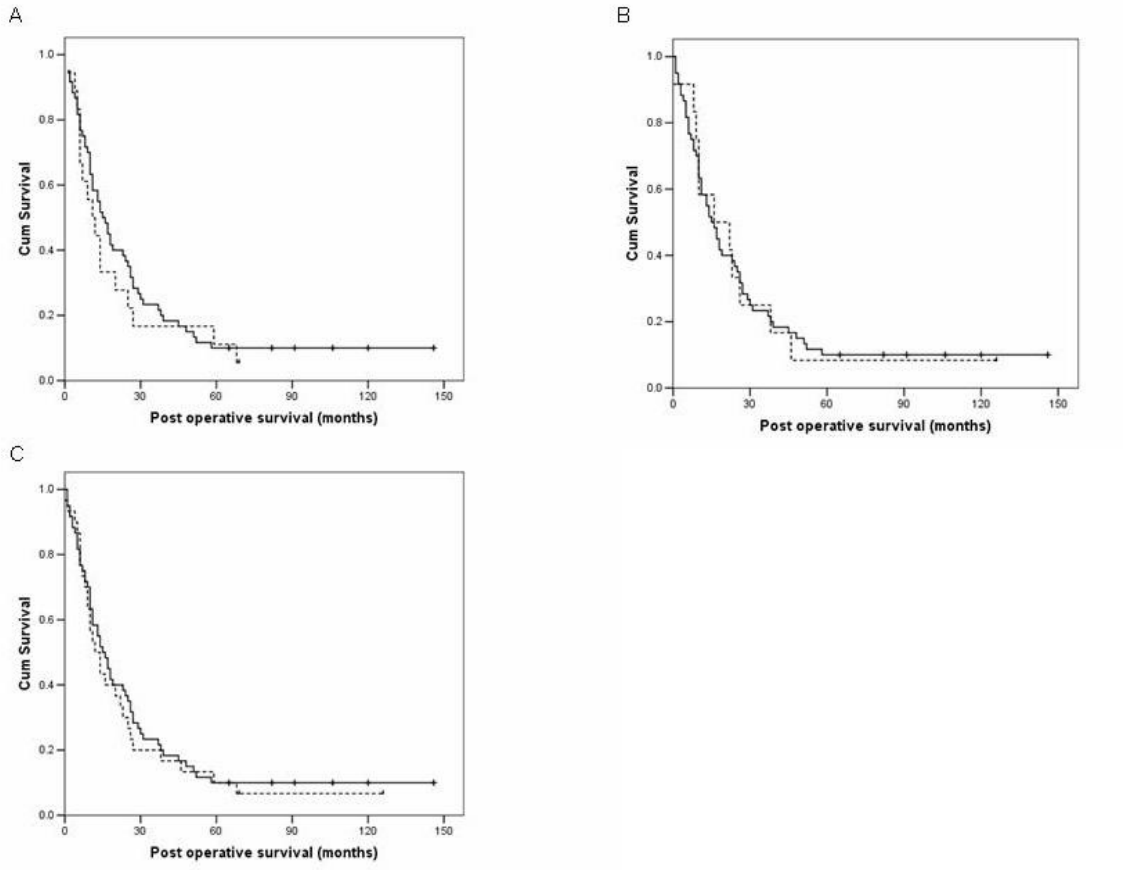
		FISH for *Topo IIα*			
		Diploid	Deleted	Polysomy 17	*Topo IIα* amplification
Sex	Male	54	2	17	11
	Female	6	0	1	1
Age at diagnosis	<65	22	1	10	3
	≥65	38	1	8	9
Tumour stage	pT1,2	10	0	3	1
	pT3	50	2	15	11
Node metastasis	pN0	14	0	5	4
	pN1	46	2	13	8
Differentiation	Poor/un-differentiated	19	2	7	3
	Well/mod differentiated	41	0	11	9
Topo IIα expression	Negative	52	2	13	7
	Positive	8	0	5	5 (p=0.03)†
HER-2 expression	0/+	59	2	17	8
	++/+++	1	0	1	4 (p<0.01)†
HER-2 amplification	No	56	2	17	5
	Yes	4	0	1	7 (p<0.01)†

† Fisher's exact test compared to diploid cells, all other comparisons were not significant.

Table 5.3 Survival analysis by Cox Regression in 92 cases of oesophageal adenocarcinoma by clinical, histopathological, and genetic status for chromosome 17, *Topo IIα* and *HER-2*.

		Hazard ratio	p-value	95% confidence interval of hazard ratio
Age		1.00	0.6	0.98-1.03
Node metastasis	0	Baseline		
	1	2.53	0.004	1.36-4.71
Stage	1/2	Baseline		
	3	1.87	0.11	0.87-4.05
Differentiation	Well/moderate	Baseline		
	Poor/undifferentiated	1.91	0.006	1.20-3.04
Topo IIα IHC	Negative	Baseline		
	Positive	0.98	0.96	0.54-1.78
Topo IIα gene	Diploid chromosome 17	Baseline		
	Topo IIα deletion	1.03	0.97	0.23-4.57
	Chromosome 17 polyploidy	1.25	0.43	0.72-2.19
	Topo IIα amplification	1.04	0.92	0.52-2.07
Combined *HER-2* and *Topo IIα* amplification	Diploid chromosome 17 or chromosome 17 polyploidy in one assay only or *Topo IIα* deletion	Baseline		
	Chromosome 17 polyploidy in both assays	3.34	0.01	1.68-6.63
	HER-2 or *Topo IIα* amplification	0.86	0.68	0.42-1.76
	HER-2 and *Topo IIα* amplification	2.28	0.05	0.98-5.27

Figure 5.4 Kaplan- Meier analysis of post-operative survival of cases of oesophageal adenocarcinoma that were diploid for chromosome 17, without *Topo IIα* amplification (solid line, + for censored cases, n=60) compared to tumours with (dotted line): **A** chromosome 17 polyploidy (n=18); **B** *Topo IIα* amplification (n=12); **C** *Topo IIα* amplification or chromosome 17 polyploidy (n=30).



5.3.5 Correlation between FISH for *HER-2 and Topo IIα*

There were discrepant results for FISH analysis of chromosome 17 diploid or polyploid status between the prior *HER-2* assay and the current *Topo IIα* assay (Table 5.4), although there was agreement in the majority of cases. Both *HER-2* and *Topo IIα* amplification were present in 7 cases (8%), a significant association as detailed in Table 5.2. There were 5 cases of single *Topo IIα* or *HER-2* amplification (5%). There was no significant association between a discrepant FISH result and *Topo IIα* assay performed on replacement specimen blocks (Table 5.5).

For Cox regression and Kaplan-Meier survival analyses, cases that were diploid for chromosome 17, or were diploid on one assay with chromosome 17 polyploidy on the other, were used as the control group. Tumours with chromosome 17 polyploidy on both FISH assays carried a higher risk of death than the control group (HR=3.34, 95% CI: 1.68 – 6.63, p=0.01) (Table 5.3). Tumours with both *Topo IIα* and *HER-2* gene amplification had a higher risk of death than the control group (HR=2.28, 95% CI: 0.98 – 5.27, p=0.05). Tumours with single amplification of *Topo IIα* or *HER-2* had no increased risk of death over the control group. Kaplan-Meier and Log-Rank analysis demonstrated a corresponding significantly worse survival in tumours with chromosome 17 polyploidy (p=0.002), and with both *Topo IIα* and *HER-2* gene amplification (p=0.046), but not in tumours with *Topo IIα* or *HER-2* gene amplification alone (Figure 5.5A-C).

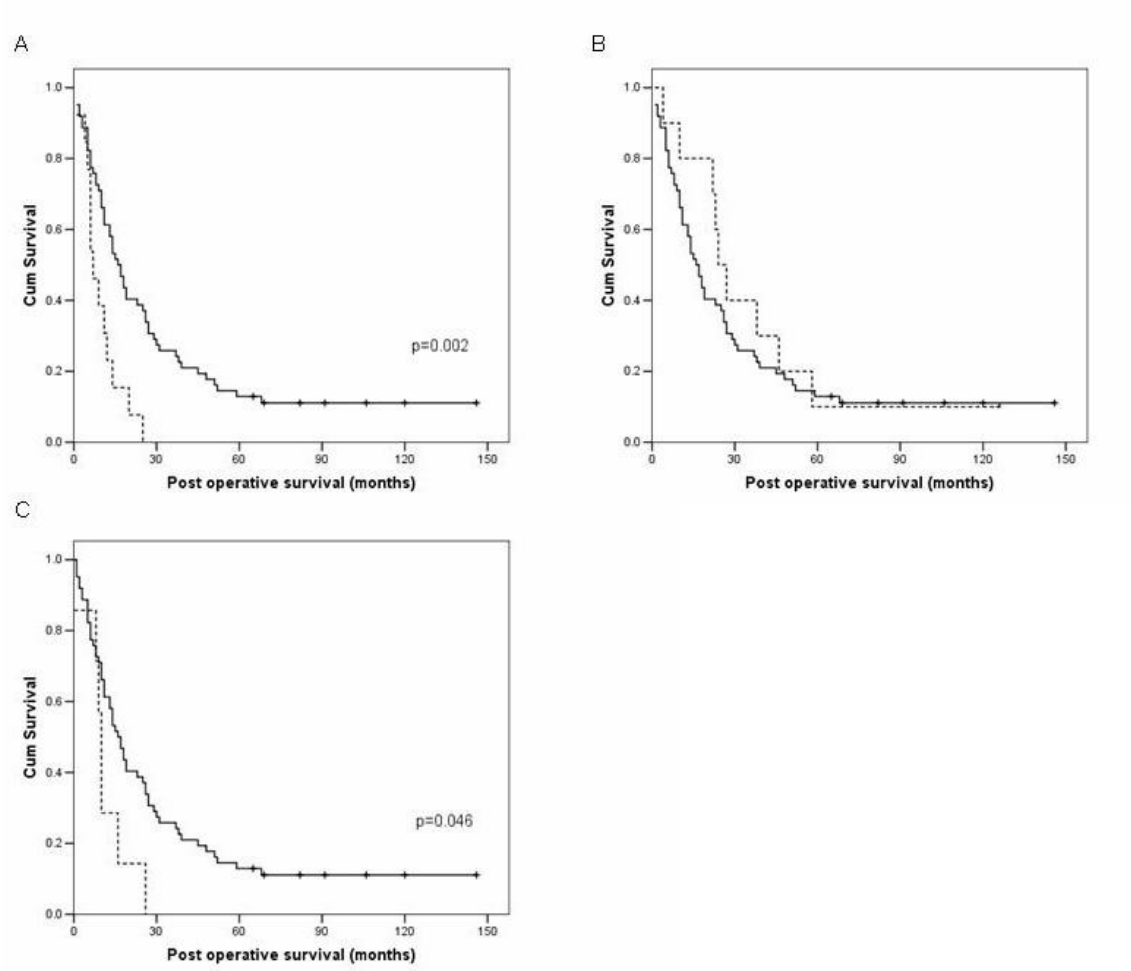
Table 5.4 Comparison of two separate FISH assay results for chromosome 17 number and *HER-2*, and chromosome 17 number and *Topo IIα* in 92 cases of resected oesophageal adenocarcinomas.

FISH result by *HER-2* assay	FISH result by *Topo IIα* assay				Total
	Deleted	Diploid	Polyploid	Amplified	
Diploid	1	47	4	5	57
Polyploid	1	9	13	0	23
Amplified	0	4	1	7	12
Total	2	60	18	12	92

Table 5.5 Agreement between FISH assays for *Topo IIα* and chromosome 17 number and *HER-2* and chromosome 17 number, by use of replacement specimen block.

Replacement block	Agreement between FISH assays		Total
	No	Yes	
Yes	1	6	7
No	23	62	85
Total	24	68	92

Figure 5.5 Kaplan- Meier and Log Rank analysis of survival of cases of oesophageal adenocarcinoma post resection analysed by two separate FISH assays for *Topo IIα* and chromosome 17, and *HER-2* and chromosome 17. Tumours that were diploid for chromosome 17 or were diploid on one assay with chromosome 17 polyploidy on the other were used as the control group (solid line, + for censored cases, n=62) compared to tumours with (dotted lines): **A** chromosome 17 polyploidy on both assays (n=13); **B** either *Topo IIα* or *HER-2* amplification (n=10); **C** both *Topo IIα* and *HER-2* amplification (n=7).



5.4 Discussion

The work presented in this chapter aimed to examine the expression of Topo IIα by immunohistochemistry and the amplification or deletion of the *Topo IIα* gene by FISH in a large series of cases of oesophageal adenocarcinoma. Furthermore, I aimed to correlate these findings with clinicopathological features and survival, as well as chromosome 17 copy number, and HER-2 over-expression and *HER-2* amplification. I have demonstrated a non-significant trend towards an increase in the mean Topo IIα labeling index with chromosome 17 polyploidy and *Topo IIα* amplification in 92 cases of oesophageal adenocarcinoma, with 20% over-expressing the protein using a cut-off of 50% of relative maximal labeling. Twelve cases (13%) showed *Topo IIα* amplification, chromosome 17 polysomy without *Topo IIα* amplification was observed in 18 cases (20%). There was no significant association between *Topo IIα* gene amplification or deletion or chromosome 17 polysomy and any clinical or histopathological criteria. *Topo IIα* amplification was significantly associated with Topo IIα protein expression ($p=0.03$). There was no significant increased risk of death with tumours with *Topo IIα* amplification, or chromosome 17 polyploidy over tumours that were diploid for chromosome 17. When combined with data on the same series from a previous study (with permission, Dr B Stacey, University of Southampton) (Stacey et al. 2006), *Topo IIα* amplification was significantly associated with both HER-2 protein expression and *HER-2* gene amplification ($p<0.01$); tumours with chromosome 17 polyploidy (HR=3.34, $p=0.01$), and co-existing *Topo IIα* and *HER-2* gene amplification (HR=2.28, $p=0.05$) had higher risk of death than the control group.

This work is the largest study of *Topo IIα* amplification in oesophageal adenocarcinoma to date. There is only one other study of HER-2/Topo IIα over-expression and *Topo IIα*/*HER-2* amplification in oesophageal adenocarcinoma employing an identical FISH detection system by Rossi *et al* (Rossi et al. 2010). From 13 cases of oesophageal adenocarcinoma, there was a statistically significant association between Topo IIα over-expression by immunohistochemistry and *Topo IIα* amplification ($p<0.001$), *HER-*

2 amplification ($p<0.001$) and HER-2 over-expression. There was also an association between HER-2 over-expression and *HER-2* amplification ($p<0.001$) and *Topo IIα* amplification ($p<0.001$). Compared to our results, they found greater protein over-expression (61 and 69% respectively), gene amplification (38% for both), and chromosome 17 aneuploidy (61%, leaving 38% diploid), although from a smaller number of cases (Rossi et al. 2010). Amplification of *Topo IIα* always occurred with *HER-2* amplification (100%) in keeping with previous work in breast cancer. In contrast, we found 7 out of 12 cases (58%) in our study where *HER-2* or *Topo IIα* amplification occurred in isolation in the remainder. This has been described in prostatic carcinoma, with *Topo IIα* amplification in 14% of all cancers, 26% in advanced cancers, roughly twice as frequent as *HER-2* amplification (Murphy et al. 2007). Rossi and colleagues found no cases of *Topo IIα* deletion, compared to two cases in our study. This is documented in 23-43% of breast cancers studied for *HER-2* gene copy number (Jarvinen et al. 1999;Slamon et al. 2001).

Our rate of amplification was comparable to the 100 gastro-oesophageal junction adenocarcinomas studied using chromosome-specific in situ hybridization (CISH) by Tanner *et al*, who showed co-amplification of *Topo IIα* in 16 cases (16%), representing 66% of the 24 cases (24%) with *HER-2* amplification (Tanner et al. 2005). Two other studies assessed *Topo IIα* amplification in gastric carcinoma. Ming Liu *et al* studied 30 cases of metastatic gastric cancer by FISH and found *HER-2* amplification in 5 (17%), with co-amplification of *Topo IIα* in 2 of these (40%) (Ming Liu et al. 2004). There was a significant positive correlation between *HER-2* amplification and both *Topo IIα* amplification and Topo IIα over-expression.

Taken together, these findings are in keeping with the observation that the genes within the amplified region at the 17q12-q21 locus, and the extent of amplification can vary from tumour type (Murphy et al. 2007). Multiple genes within this amplicon have been studied: in gastric cancer using comparative genomic hybridization on a cDNA microarray, revealing increased copy numbers of 11 known genes, including

Topo IIα and *HER-2*, which were also over-expressed on gene expression analysis (Varis et al. 2002); a larger sample of oesophago-gastric tumours using FISH on a tissue microarray demonstrated increased copy number of 8 genes, with over-expression of Topo IIα mRNA by quantitative real-time reverse transcription-PCR (Maqani et al. 2006). The amplification of these genes is variable, and the over-expression can be independent (Varis et al. 2004). The determinants of this variability are yet to be elucidated.

In using a labeling index to determine Topo IIα expression, the inaccuracy of an ordinal categorization was avoided. It did, however, result in a fairly linear increase in the degree of expression with no clear pattern, so an arbitrary cut-off was made to allow statistical comparison. With no FDA-approved method to provide a consensus approach, a variety of different methods have been used in the literature. Rossi *et al* describe positive immunostaining was present when clearly recognized nuclear brown staining was seen in 10% of tumour nuclei (with comparison drawn to the criteria for positive p53 staining criteria) (Rossi et al. 2010;McCarthy et al. 2002). However they report the quartile ranges of the percentage labeling index in their results, but make no comment as to why this is the case. All 13 adenocarcinoma cases would be positive using such a low cut off, reducing the significance of any correlation with gene amplification. Our data support this finding. Other investigators have used as low a threshold as 5% labeling index to indicate positive staining in prostatic carcinoma, but a study of 11 cases of Barrett's with HGD used a scoring system of negative-weak (0-10%), moderate (11-50%), strong (>50%) (Murphy et al. 2007;Sabo et al. 2008).

The prevalence of Topo IIα over-expression overall was higher than the prevalence of *Topo IIα* amplification, although not all the tumours with *Topo IIα* amplification displayed Topo IIα over-expression. This is in keeping with our HER-2 work in this series, but differs from studies in breast carcinoma in which the proportion of tumours showing HER-2 over-expression was greater than the proportion showing HER-2 gene amplification – with almost all HER-2 3+ positive tumours but only 12% of HER-2 2+

positive tumours (on immunohistochemistry) showing HER-2 gene amplification in two large series (Ellis et al. 2005;Mrozkowiak et al. 2004;Varshney et al. 2004;Perez et al. 2002). The reason for this difference is unclear but it is possible that suboptimal antigen preservation in some of the oesophageal adenocarcinomas examined may have led to a reduction in the Topo IIα labeling index. Despite this, we found a correlation between Topo IIα over-expression and *Topo IIα* amplification, analogous to the situation in breast cancer. Breast cancers that possess *HER-2* amplification, *Topo IIα* amplification or deletion is present in 41-44% and 23-43% respectively (Jarvinen et al. 2000;Jarvinen et al. 1999;Slamon et al. 2001). This is somewhat in keeping with our results of 7 from 12 cases (58%) with *Topo IIα* co-amplification with *HER-2* amplification. Neither of the two *Topo IIα* deleted cases had *HER-* amplification. As a technique, FISH is liable to insufficient hybridization, with factors such as section thickness, incubation time and data evaluation can affect results. Counting the fluorescent signals was particularly difficult when nuclei overlap in solid organ, paraffin-embedded specimens, with the possibility that only a proportion of the nucleus may be present. This is partly corrected by using the breast cancer derived definition of a gene copy number:centromere number ratio of $\geq$2.0, although in this setting the majority of amplified cases had ratios $>$5.0. The greatest ratio observed in our study was 5, with a ratio of 2 in the majority. This lower magnitude of amplification is consistent with other studies in oesophageal adenocarcinoma and colorectal cancer, and the possible impact this may have on statistical analysis is unknown (Rossi et al. 2010;Al-Kuraya et al. 2006).

The Vysis PathVysion® FISH kit that we used enabled differentiation between cells with pure *Topo IIα* amplification and those showing chromosome 17 polysomy. The prevalence of amplification was 13%, in agreement with the 16% of Tanner's gastro-oesophageal adenocarcinoma cohort. However, we found 12% of tumours possessed *HER-2* amplification compared to 24% in Tanner's study, somewhat lower than previous studies using dual colour FISH where gene amplification was present in 32% (n=24), 35% (n=25), 38% (n=13), and 83.3% (n=6) of Barrett's-associated

adenocarcinomas respectively (Tanner et al. 2005;Walch et al. 2004;Walch et al. 2001;Rossi et al. 2010;Walch et al. 2000a). Furthermore, I identified cases with *Topo IIα* amplification without *HER-2* amplification. Tanner did not find this in their initial study of 131 gastric adenocarcinomas, and then only studied the *HER-2* amplified gastro-oesophageal for *Topo IIα* amplification. This was also not the case in Rossi's most recent work (Rossi et al. 2010). Interestingly, we found 5 tumours with immunohistochemical evidence of Topo IIα over-expression associated with chromosome 17 polysomy rather than specific *Topo IIα* gene amplification. Walch *et al* found chromosome 17 polysomy without *HER-2* amplification in 52% of Barrett's associated adenocarcinomas, with normal or moderately increased mRNA expression and no or weak immunopositivity (Walch et al. 2001). Our series contained a higher proportion of tumours diploid for chromosome 17 (65%) than the 0-40% previously reported (Beuzen et al. 2000). There were two cases of mono-allelic deletion in the setting of chromosome 17 trisomy. This has been described in breast cancer, although has not been previously observed in oesophageal adenocarcinoma (Bouchalova et al. 2006).

Some studies have included assessment of Barrett's oesophagus, low and high grade dysplasia to assess at which point these genetic aberrations are occurring in the metaplasia-dysplasia-adenocarcinoma sequence. Walch and colleagues demonstrated that polysomy 17 with no *HER-2* amplification was present in 4 of 8 cases of LGD, 5 of 13 areas of HGD, and in 13 of 25 cases of adenocarcinoma using the same Vysis chromosome 17 FISH probe as we used (Walch et al. 2001). Again using the same FISH kit, Rossi *et al* found aneusomy 17 without *HER-2* or *Topo IIα* amplification in none of the 18 cases of Barrett's (with no cases of *HER-2* or *Topo IIα* amplification either), 1 of 5 cases of LGD, 3 of 8 cases of HGD, and 8 of 13 cases of adenocarcinoma (Rossi et al. 2010). Furthermore, there was a significant association with chromosome 17 aneusomy and Topo II protein expression by immunohistochemistry ($p=0.03$), as well as HER-2 ($p=0.02$). A study that looked at a number of different chromosomes by CISH found a progressive increase in chromosomal numerical alterations with advancing

degrees of dysplasia in gastric and oesophageal tumours (Beuzen et al. 2000). Furthermore, trisomy of chromosome 17 (and 7, 8, and 11) was observed in some samples of Barrett's mucosa without dysplasia, and appeared to be an early event in Barrett's carcinogenesis. Increased expression of Topo IIα mRNA by RT-PCR in laser capture microdissected and protein by IHC on paraffin-embedded biopsy specimens was shown in HGD (Sabo et al. 2008). It is widely accepted that the development Barrett's oesophagus is linked to gastro-oesophageal reflux, and it has been demonstrated in vitro that acid and components of the refluxate can induce DNA damage, possibly in a topoisomerase II-dependent manner (Jolly et al. 2004;Xiao et al. 2003). A further study suggested that the over-expression of HER-2 in dysplastic Barrett's may predict early transition to adenocarcinoma (Rossi et al. 2009). Intense HER-2 expression has been described in oesophageal adenocarcinomas with gene amplification, while moderate and weak protein expression has been identified in tumours with chromosome 17 polysomy alone and in other tumours showing no specific oncogene amplification (Walch et al. 2001;Geddert et al. 2002). As mentioned previously, this has been observed in many tumour types, suggesting that there may be regulation of protein expression at the transcriptional or post-transcriptional level, as well as due to oncogene amplification (Geddert et al. 2002).

There was a discrepancy between the number of chromosome 17 centromere signals by the two FISH assays we performed in 15 cases. This could have resulted from the above mentioned factors that can influence FISH results. The use of both kits on sequential sections, or the use of the Vysis tricolour FISH probe kit would have overcome this. Given the amount of tissue sectioning from each specimen block, an alternative explanation for this heterogeneity involves clonal expansion of each tumour, with different genetic alterations in different areas of the tumour. This is thought to occur either from the traditional founder mutation in a single progenitor cell theory, with clone bifurcation responsible for the heterogeneity (Maley et al. 2004b); or the more recently proposed theory of mutation of multiple progenitor cells situated in the oesophageal gland squamous ducts located throughout the

oesophagus, resulting in multiple independent clones that evolve separately (Leedham et al. 2008). Multiple sections will result in a significant "traverse" through the paraffin block, although the remaining presence of tumour was confirmed on the accompanying immunohistochemistry slide.

The prognostic significance of *Topo IIα* and *HER-2* gene amplification in oesophageal adenocarcinoma remains uncertain. Our study was the first to investigate the possible relationship between Topo IIα/ *Topo IIα* expression/amplification and outcome. Previous studies looking at survival have focused on HER-2 alone. I found that only by combining the survival data for tumours with *Topo IIα* and *HER-2* gene amplification or chromosome 17 polysomy alone resulted in a statistically significantly greater risk of death compared to patients with disomy and no evidence of amplification of either gene. This may represent a sample size effect but may also suggest that both groups of tumours are more aggressive. The only previous study to investigate the prediction of poor survival looked at *HER-2* amplification in Barrett's oesophagus associated adenocarcinoma, by Brien and co-workers. They found that *HER-2* amplification was significantly associated with shortened survival (Brien et al. 2000). However, the FISH technique employed in this study used a locus-specific probe only. Dual-colour FISH using DNA probes complementary to *HER-2* and the chromosome 17 centromere are required to distinguish *HER-2* amplification from chromosome 17 polysomy, although their findings support the view that tumours with chromosome 17 polysomy alone and *HER-2* amplification are more aggressive. In keeping with the findings of our work, there was no significant correlation with any of the pathological features of the tumour and the presence of *HER-2* amplification (Brien et al. 2000).

Previous studies investigating survival in oesophageal adenocarcinoma and HER-2 over- expression by immunohistochemistry have produced variable results. Two studies involving 66 and 80 cases of oesophageal adenocarcinoma found that HER-2 over- expression was associated with a significantly poorer prognosis (Flejou et al. 1994;Nakamura et al. 1994). Conversely, Hardwick and co-workers found no

significant correlation between HER-2 over- expression and prognosis in 127 cases, and in 42 patients with oesophageal adenocarcinoma Duhaylongsod *et al* actually found that HER-2 over-expression was associated with improved 5-year survival (Hardwick et al. 1995;Duhaylongsod et al. 1995). In this later study, all cases had received preoperative chemoradiotherapy, of which the impact on *HER-2* amplification and expression is at present unknown. In agreement with Brien *et al*, such cases were excluded from our cohort (Brien et al. 2000).

The immunohistochemical and FISH methods that we used are designed primarily to predict response to trastuzumab therapy rather than to determine prognosis. If oesophageal adenocarcinoma is biologically comparable to breast carcinoma in this regard, it is possible that oesophageal adenocarcinomas showing HER-2 over-expression or gene amplification (i.e. HER-2 'positive') using these methods might also respond to trastuzumab therapy – in the adjuvant, neoadjuvant or palliative setting. The encouraging results of the ToGA trial in gastric carcinoma, with improved overall and progression free survival with the addition of trastuzumab to standard chemotherapy, suggest that this agent may be helpful in oesophageal adenocarcinoma (Van Cutsem et al. 2009). Limited preliminary data of the use of trastuzumab in two cases of HER-2 positive oesophageal adenocarcinoma suggested down regulation of both HER-2 and Topo IIα by IHC and induction of apoptosis after six months of therapy (Villanacci et al. 2008). The amplification of Topo IIα may indicate that an oesophageal adenocarcinoma may better respond to anthracycline-based chemotherapy, as in breast cancer, although the mechanism remains unclear (Harris et al. 2009). Our findings of cases of gene amplification without protein over-expression, and vice-versa, as well as protein over-expression in the setting of chromosome 17 polyploidy support the theory of post-transcriptional and post-translational controls that may determine response to chemotherapeutic agents. Indeed, a large recent study of 782 node-negative breast carcinomas that did not receive systemic therapy prior to surgery, and 80 cases that received epirubicin and cyclophosphamide, revealed no correlation between *Topo IIα* amplification and protein expression, and only a

borderline association with gene expression (Brase et al. 2010). Topo IIα RNA levels were very significantly associated with protein expression, metastasis-free interval, and complete remission in those who received chemotherapy. Our finding of an increased risk of death with combined gene amplification and chromosome 17 polyploidy are in keeping with the fact that other potential oncogenes lie within the chromosome 17 amplicon. Two candidates are the human growth factor receptor-bound protein 7 (GRB7) and DARPP-32, and its truncated isoform t-DARPP (Maqani et al. 2006;Dahlberg et al. 2004;Walch et al. 2004). GRB7 mRNA over-expression has been documented in 44% of 32 human oesophageal carcinomas, interestingly without gene amplification, and co-expression with HER-2 or EGFR was present in 31% and was significantly related to extramucosal tumour invasion compared to sole expression (Tanaka et al. 1997). Walch *et al* demonstrated co-amplification and co-expression of GRB7 and HER-2 in 32% of 32 oesophageal adenocarcinomas, with correlation at the DNA and mRNA level by PCR, extending to a subset of HGD, also suggesting a role in tumour invasion (Walch et al. 2004). t-DARPP is an anti-apoptotic protein that has been shown to be expressed in upper gastrointestinal cancers, and activates the Akt survival signal pathway that has been shown to mediate trastuzumab resistance in breast cancer (El-Rifai et al. 2002;Belkhiri et al. 2005;Belkhiri et al. 2008;Hamel et al. 2010).

Chromosomal aneuploidy is commonly observed in oesophageal adenocarcinoma, and seen late in tumour progression (Maley et al. 2004a). The association with increased risk of death may therefore indicate a tumour that has acquired more sequential genetic changes that confer more aggressive behaviour, rather than a causal role (Renan 1993). Chromosomal aneuploidy has been considered a secondary event to uncontrolled proliferation (Schindlbeck et al. 2010), although recent work suggests it may be a primary event, with deregulated expression of chromosome passenger proteins, such as Aurora kinases or Survivin that are involved in cell cycle progression (Nguyen & Ravid 2006). Further work by the same investigators revealed deregulated Aurora kinase B (involved in chromosome condensation and mitotic spindle assembly)

induces tetraploidy in a murine xenograft model (Nguyen et al. 2010). Comparative chromosome hybridization performed on DNA from tetraploid cell-induced tumours indicated amplifications and deletions throughout the genome (although not in *Topo IIα*), in tumour-promoting or tumour-suppressing genes respectively, the genetic instability that leads to tumour formation. Recent work suggests that Topo II ensures proper sister chromatid separation, and allows proper activation of Aurora kinase B (Coelho et al. 2008), suggesting a possible positive feedback mechanism. Aurora kinase A over-expression also causes aneuploidy, and is frequently over-expressed in oesophageal adenocarcinoma and has been demonstrated in Barrett's carcinogenesis (Zhou et al. 1998;Dar et al. 2008;Rugge et al. 2010).

The results of our study, and the possibility that *HER-2* and *Topo IIα* amplification indicate possible response to trastuzumab and anthracycline-based chemotherapy respectively, suggest that evaluation of these biomarkers may be useful to profile patients with oesophageal adenocarcinoma prior to entry into a clinical trial of trastuzumab and a Topo IIα-specific chemotherapeutic agent.

5.4.1 Future Work

It would appear that there are a number of avenues for further research to investigate the prognostic significance, mechanism of action and prediction of response to anthracycline-based chemotherapy of Topo IIα amplification and over-expression of its protein product in oesophageal adenocarcinoma. Despite the work performed in the breast cancer field, a consensus on the prognostic significance and prediction of response to chemotherapy has yet to be reached (Depowski et al. 2000;Callagy et al. 2005;Coon et al. 2002;Jarvinen et al. 2000;Di Leo et al. 2002;Kawachi et al. 2010;Harris et al. 2009;Mueller et al. 2004). This question is only just beginning to be asked in gastric and oesophageal adenocarcinoma. Large scale, prospective randomised controlled trials will need to be performed to answer this question. Given the experience in breast cancer studies, oesophageal tumours should be profiled for

HER-2 and *Topo IIα* gene copy number by FISH and expression by immunohistochemistry, then randomized to standard chemotherapy, or standard chemotherapy plus any number of potential new Topo II poisons being developed. Response to adjuvant therapy and survival can then be studied. An FDA approved method for the grading of Topo IIα expression by immunohistochemistry, as in HER-2 testing, will standardize assays and allow easier comparison between trials. The common finding of discrepancy between gene amplification and protein expression, in a number of tumour types, suggest that further dissection of the post-transcriptional and post-translational controls that determine Topo IIα expression is required.

The mechanism of action of Topo IIα over-expression and responsiveness to chemotherapeutic agents demands further attention. Limited data in oesophageal adenocarcinoma suggested increased apoptosis with down-regulation of HER-2 and Topo IIα after trastuzumab therapy (Villanacci et al. 2008), combined with a more recent study in colorectal carcinoma suggest an inhibition of apoptosis to explain increased tumour survival (Coss et al. 2008), suggest further study of the apoptotic pathways involved. Oesophageal adenocarcinoma cell lines, and tissue explant models of tumours known to possess *Topo IIα* amplification could be used, and techniques such as the TUNEL microscopic technique or caspase assays employed. The mitogen-activated protein kinase (MAPK) pathways and p53 have already been implicated in evading the apoptotic process in Barrett's carcinogenesis (Morgan et al. 2004). Genetic silencing of p53 by RNA interference would determine whether apoptosis in the tissue model was dependent on this key molecule, and transfection of cell lines to over-express the anti-apoptotic Bcl-2 could be used to investigate its role. This work would overlap with further investigation of the roles played by all the genes in the chromosome 17q12-q21 amplicon. As mentioned above, t-DARPP is an anti-apoptotic protein that activates the Akt survival signal pathway, the encoding gene lies within this amplicon. Microarray analysis of gene expression changes in Barrett's oesophagus and adenocarcinoma cell lines and tissue from laser capture microdissected tissue will continue to highlight potential candidate genes, and focus

attention on further investigation of potentially useful biomarkers (Morgan et al. 2004;Sabo et al. 2008;Albrecht et al. 2004). The ultimate outcome for this research will allow a tailored tumour-specific approach to future treatment regimens for oesophageal adenocarcinoma, and potentially in the pre-malignant phase of Barrett's oesophagus (Fitzgerald 2006).

6 SUMMARY AND CONCLUSION

6.1 Summary

Barrett's oesophagus offers the opportunity to study the metaplasia-dysplasia-adenocarcinoma sequence; with relatively easy, endoscopic access to quantify and biopsy the area of interest. Whilst a number of risk factors have been identified and the association with gastro-oesophageal reflux disease widely accepted, Barrett's has been found in a proportion of patients without symptoms (Ward et al. 2006). Furthermore, the place of endoscopic screening or surveillance for Barrett's oesophagus remains the topic of much debate (Moayyedi 2004;Sampliner 2004), with little supportive evidence and argument over its cost effectiveness (Sharma et al. 2004), but is still practiced widely (Falk et al. 2000). The first large, prospective, multicentre randomised controlled trial is currently recruiting in the UK to answer the question whether the benefits of two-yearly endoscopic surveillance outweighs the risks compared with endoscopy at need only (Barrett's Oesophagus two yearly Surveillance versus no surveillance: a randomised controlled trial to estimate effectiveness and cost effectiveness Study, BOSS) (Nicholson & Jankowski 2009). However, the role of endoscopy in the diagnosis and assessment of Barrett's oesophagus is unchallenged at present, and therefore a standardized endoscopic reporting system will allow more robust assessment of disease progression and comparison between clinical trials. The Prague C & M criteria are the first attempt at such standardization, and proved to be reliable and simple to use when presented. As straightforward as it seems, the anatomical landmarks employed are still open to interpretation by the individual endoscopist, with the measurement error of using an imperfect "rule" (an endoscope with 5cm incremental markings), as well as the patient factors of patient movement during the procedure, respiration and the distending effect of air insufflation. Numerous assessors were used in the validation of the Prague criteria to improve the statistical power of the inter-rater agreement. This necessitated the use of video clips, and an entirely artificial setting. My work is the first live-endoscopy validation of the Prague C & M criteria. Perhaps the statistical power was reduced by the maximum of two assessors, but by using multiple techniques to analyse the data, there was a

clinically relevant measurement error. It is that "clinical relevance" that is most important; any future trial of drug therapy, particularly of chemopreventative agents to prevent the progression through to dysplasia at the earliest stage, may well look to report a reduction in the length of Barrett's oesophagus as a primary end point. Any reduction in length will have to be above a predetermined level to account for this important observation. The interim results of the AspECT trial are due to be reported in 2011 and it will be fascinating to interpret the results in light of our findings (Das et al. 2009).

The results of our studies into the expression and activity of 5-LO and its activating protein FLAP in Barrett's metaplasia, dysplasia and adenocarcinoma also have important implications for the analysis of the results of the AspECT trial. This is particularly relevant given the two potential mechanisms that NSAIDs could potentiate the pro-inflammatory effects of LTs, the "PGE_2 brake" and "shunting" hypotheses (Sampson & Holgate 2004;Szczeklik 1990;Szczeklik 1995;Sestini et al. 1996). The limbs of the AspECT trial involve the proton pump inhibitor, esomeprazole either alone or in combination with aspirin therapy. Such "unopposed" NSAID activity may well lead to increased production of LTs, and whilst an increase in tumour incidence in the aspirin group is very unlikely, the possibility that further chemoprevention could be offered by inhibiting the production of LTs should not be overlooked. A recently reported trial of a COX-2-selective inhibitor, celecoxib, in patients with Barrett's and LGD or HGD did not show a protective effect against disease progression (grade of dysplasia, total surface area, prostaglandin levels, COX-2 mRNA, or tumour-suppressor gene methylation) (Heath et al. 2007). Moreover, the impressive reduction in tumour incidence in a rat model of oesophageal adenocarcinoma by the specific 5-LO inhibitor, zileuton, and the even greater effect observed in combination with celecoxib, suggest further investigation of combination therapy in human trials would be beneficial (Chen et al. 2004).

For many types of carcinoma, the era of tumour-specific, tailored therapy is becoming a reality. This has been consequent on the identification of biomarkers that can be tested easily and reliably, and indicate either a poor prognostic group worthy of more intensive general therapy, or surrogate markers for/definitive targets of the new, highly specific biological agents (Glynn et al. 2010). Topo IIα has received much attention in the breast cancer field, with increasing interest in gastrointestinal cancers. I have presented the largest series, including outcome data, of the amplification and expression of *Topo IIα*/Topo IIα in oesophageal adenocarcinoma. The rate of amplification was not high in our cohort, but when combined with *HER-2* and chromosome 17 polyploidy there was an increased risk of death. This data would support a trial of trastuzumab alone or in combination with an anthracycline in oesophageal adenocarcinoma, especially given promising early results from trials in gastric cancer. As evidence regarding each biomarker expands, a panel of biomarkers may be developed in the future, to profile tumours and guide therapy. Indeed, novel non-endoscopic, minimally invasive techniques to diagnose Barrett's oesophagus such as the "Cytosponge" are already being developed (Lao-Sirieix et al. 2009;Kadri et al. 2010). This will allow the profiling to be extended to the pre-malignant phase, perhaps assisting in selecting the patients with Barrett's that are at risk of malignant transformation. This will help guide both therapy and rationalise the current programme of endoscopic surveillance for all, focusing on those at greatest risk, and allowing those with the lowest risk to be reassured and discharged from follow up.

6.2 Conclusion

All three aspects of Barrett's oesophagus that have been studied are fundamental to the call for improved risk stratification (Shaheen & Richter 2009), and invite further research to expand the current findings. Ultimately this will aim to improve our ability to predict the small, but growing, numbers of patients who progress from metaplasia to dysplasia and finally adenocarcinoma, from the enormous number of patients with Barrett's oesophagus at risk. There may be opportunities to modify the progression of the disease along this sequence with drugs, and offer targeted therapy to improve the presently poor outcome from current treatments.

7 APPENDIX

7.1 Materials

10x Tris Buffered Saline (TBS)	25ml 2 M Tris-HCl pH 8
	150ml 5 M NaCl
	325ml ultrapure water
10x Phosphate Buffered Saline (PBS)	80g NaCl
	2g KCl
	14.4g Na_2HPO_4
	2.4g KH_2PO_4
	Made up to 1000ml with ultrapure water
20 x SSC	175.3g NaCl
	88.2g Sodium citrate
	pH to 7 with HCl
	Made up to 1000ml with ultrapure water

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