The Incidence and pathogenesis of 'recurrent' Barrett's metaplasia following oesophagectomy (Neo-Barrett's)

Lorna Jane Dunn

A Thesis Presented For the Degree of
Doctor of Medicine

University of Newcastle-upon-Tyne
Institute of Cellular Medicine

February 2013

Thesis Abstract

Aims

Columnar metaplasia in the oesophageal remnant occurring after subtotal oesophagectomy (neo-Barrett's) has been proposed as a human model for the development of Barrett's oesophagus. This study aimed to assess the incidence of this phenomenon and it's accuracy as a model as well as looking for evidence of field cancerisation in the oesophagus.

Methods

Patients underwent prospective endoscopic evaluation having previously undergone oesophagectomy. The presence or absence of columnar epithelium above the surgical anastomosis was noted and biopsies taken. Specimens were stained using H&E and, where consent was granted, with immunohistochemical stains for proteins which have a well described expression pattern in Barrett's oesophagus. Tumours and adjacent Barrett's oesophagus from patients who subsequently developed neo-Barrett's were screened for genetic mutations. Where these were present, subsequent neo-Barrett's samples were evaluated for the presence of these mutatations

Results

Of 126 eligible patients, 45 (36%) had confirmed neo-Barrett's. Median time from surgery was greater for patients with neo-Barrett's (5.7 vs 2.2yrs, $p<0.001$). There were no cases of dysplasia. Non-intestinalised columnar epithelium occurred earlier than neo-Barrett's with specialised intestinal metaplasia. Surgery for dysplastic Barrett's or adenocarcinoma was associated with a similar prevalence of neo-Barrett's to other indications (41% vs 27%, $p=0.157$). 37 samples underwent molecular analysis. Typical, Barrett's like CK7/20 staining pattern was present in 23 cases (62%). Chromogranin A and trefoil factors 1 and 2 were were present in all cases. TFF3 expression was significantly associated with increasing time from surgery (median 8.1yrs vs 3.4yrs, $p=0.004$). Genetic mutations identified in the resection specimen were not present in the neo-Barrett's tissue.

Conclusions

Columnar metaplasia is common following oesophagectomy. Cellular protein expression is similar to that of sporadic Barrett's suggesting this is an accurate model. Presence of intestinal metaplasia and TFF3 expression appear to represent later stages in the development of Barrett's. No evidence of field cancerisation was found.

Acknowledgements

This work was undertaken whilst the author was a Research Fellow in the Northern Oesophago-gastric Cancer Unit at the Royal Victoria Infirmary, Newcastle-upon-Tyne.

I wish to thank Mrs Sharon Johnson, Mrs Helen Jaretzki and Mrs Angela Tait who tirelessly maintain the prospective patient database which provides valuable information for projects of this type. I also wish to thank Mr Thomas Ness of the department of pathology at the Royal Victoria Infirmary for his help retrieving and preparing specimens.

The work looking at genetic mutations in tumours and neo-Barrett's was undertaken at the Cancer Research UK laboratory, Lincolns Inn Fields, London. This was a collaborative project undertaken in association with Dr Shabbudin Khan with the practical laboratory work divided equally between ourselves. The written work included in this thesis is entirely that of the author. I am indebted to Dr Stuart Macdonald who supervised this work and provided help and inspiration for this part of the project.

I wish to thank Professor A Burt for his help, particularly with the pathology and immunohistochemisty aspects of this study and Professor S M Griffin and Professor J Jankowski who helped to devise the project and provided valuable help and enthusiasm, together with that received from Mr J Shenfine.

Table of Contents

List of tables

List of figures

Chapter 1. Introduction

1.1 Barrett's Oesophagus

Barrett's oesophagus is a condition in which the normal squamous mucosa of the oesophagus is replaced by a metaplastic columnar epithelium. Over recent years there has been a move away from this eponymous description towards the descriptive terminology 'Columnar Lined Oesophagus' (CLO). The importance of this condition relates to the associated increased risk of oesophageal adenocarcinoma. Over the past three decades, the incidence of oesophageal adenocarcinoma has been increasing faster than that of any other major malignancy in the western world. This is felt to represent a true increase in disease burden, unexplained by reclassification or changing diagnostic practice(Pohl and Welch, 2005).

1.2 Historical background

Barrett's oesophagus owes its name to Norman Barrett (1903-1979) an Australian born surgeon working in England and president of the British Society of Thoracic and Cardiovascular Surgeons of England. There can however be few eponymous conditions with quite such a complex and intriguing history.

The first descriptions of this condition pre-date Barrett's seminal paper by almost fifty years. Tileston (1906) described cases of oesophageal ulcers found at autopsy. He noted the 'close resemblance of the mucous membrane about the ulcer to that normally found in the stomach'. Barrett's original paper (Barrett, 1950) described several cases of oesophageal ulcers which were surrounded by columnar epithelium. In this paper he incorrectly hypothesised that this columnar lined organ was in fact stomach which had been pulled up into the mediastinum as a result of a congenitally short oesophagus.

Allison and Johnstone (1953) reported the cases of seven patients with columnar lining of what they correctly recognised as being lower oesophagus rather than intra-thoracic stomach. They also described the presence of goblet cells in one patient, the hallmark of specialised intestinal metaplasia. Barrett came to accept Allison and Johnstone's theory that the condition represented columnar lined oesophagus and in 1957 published a further paper recognising this(Barrett, 1957). The next key debate was whether this columnar epithelium was congenital or acquired. Barrett had concluded that it was a congenital

condition resulting from ‘a failure of the embryonic lining of the gullet to achieve normal maturity’, a view which was widely accepted. Hayward (1961) challenged this hypothesis in a landmark paper, postulating that columnar lined oesophagus was a metaplastic condition, acquired as a result of gastro-oesophageal reflux. Evidence for this theory came from a canine model(Bremner et al., 1978). This experiment showed that excision of the lower oesophageal lining was followed by the return of squamous epithelium unless acid reflux was introduced, in which case a columnar epithelium resulted.

It is universally accepted today that Barrett’s or columnar lined oesophagus is a metaplastic condition acquired due to the injurious effects of acid and bile reflux.

1.3 Diagnosis of Barrett’s Oesophagus

The diagnostic criteria for Barrett’s oesophagus have changed several times and differences remain between European and American guidelines. This inconsistency is important, not only for clinicians and patients, in part it explains the variation in reported rates both of Barrett’s and in the associated risk of malignancy.

1.3.1 Endoscopic features of Barrett’s

It is possible to identify columnar epithelium during endoscopic examination of the upper gastro-intestinal tract. It appears redder and more velvet-like in texture than squamous epithelium which has a pale, almost glossy appearance. Where the squamo-columnar junction occurs above the gastro-oesophageal junction, a segment of Barrett’s is present.

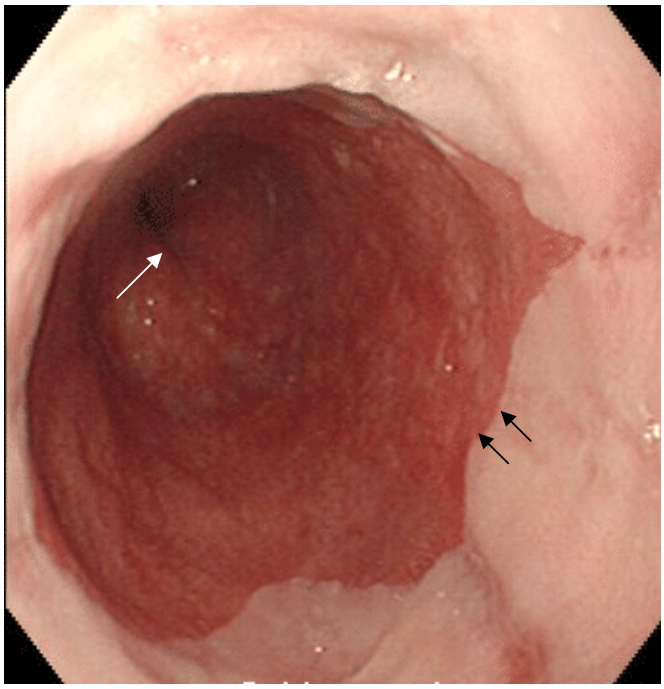


Figure 1.1: Endoscopic view of a segment of Barrett's oesophagus. Single white arrow shows gastro oesophageal junction (GOJ), double black arrows show squamo-columnar junction proximal to GOJ

The difficulty in diagnosing Barrett's at endoscopy relates to the difficulty in identifying the precise location of the gastro-oesophageal junction. The lower oesophageal sphincter can be readily identified during oesophageal mannometry but this is rarely carried out prior to endoscopy. In the absence of this precise measurement various surrogates are used. There is often a distinct 'flaring out' as one enters the stomach but this feature is lost in the presence of a hiatus hernia. The accepted method of identifying the gastro-oesophageal junction is, therefore, to note the position of the most proximal gastric folds. Even this method is somewhat unsatisfactory as over insufflation by the endoscopist will distend the lumen and obliterate the folds resulting in the position of the GOJ being incorrectly identified.

When a long segment of columnar epithelium is present the diagnosis is obvious. However when shorter segments are present the issue of precise location of the GOJ becomes critically important. The endoscopist may incorrectly identify columnar epithelium in the gastric cardia as columnar lined oesophagus, especially in the presence of a hiatus hernia. In an effort to prevent such false positive diagnoses, early investigators set an arbitrary

requirement of a 3cm segment of columnar epithelium in order to make the diagnosis. Subsequent discovery that adenocarcinoma could arise in shorter segments of columnar epithelium led to the recognition of so-called 'short-segment Barrett's oesophagus' with a length of <3cm(Schnell et al., 1992). In recent years this classification into short or long segment disease has fallen by the wayside. Whilst there is evidence to show an association between segment length and malignant progression, a diagnostic cut-off at 3cm is not clinically relevant(Weston AP et al., 2004, Rudolph RE et al., 2000). Both the British Society of Gastroenterology and the American College of Gastroenterology no longer include segment length in their definitions of Barrett's oesophagus.

The Prague C and M criteria (Prateek et al., 2006) have been proposed as a method of standardising endoscopic descriptions. Segments of Barrett's are described in terms of circumferential (C) and maximal (M) length from the gastro-oesophageal junction. These have been demonstrated to have excellent inter-observer reliability between expert endoscopists with a specialist interest in Barrett's and there is some evidence that this is reproducible for trainee endoscopists (Vahabzadeh et al.). There is however no consideration of islands of columnar mucosa in these criteria.

1.3.2 Histological features of Barrett's

Histology alone rarely proves diagnostic for Barrett's oesophagus. Only biopsies showing native oesophageal structures with juxtaposition to metaplastic glandular mucosa provide definitive histological proof of Barrett's (British Society of Gastroenterology guidelines, 2005). This situation is rare and accurate diagnosis is therefore best achieved with histological corroboration of endoscopic findings. For this reason it is essential that precise descriptions of the site of biopsies are available to the pathologist.

In their seminal paper Paull et al (1976) used manometric techniques to prove the oesophageal nature of biopsy samples. They demonstrated a spectrum of epithelial patterns in patients with columnar lined oesophagus. Three distinct types of columnar epithelium are described (figure 1.2).

1. Gastric fundic type epithelium with parietal and chief cells
2. Junctional or Cardiac type epithelium with cardiac mucus glands
3. Specialised columnar epithelium with a villiform surface mucus glands and intestinal type goblet cells (also termed specialised intestinal metaplasia)

Gastric fundic and junctional type epithelium can be indistinguishable from the normal lining of the gastric fundus and cardia and hence are not diagnostic for columnar metaplasia unless the biopsies are known to have been taken from the oesophagus.

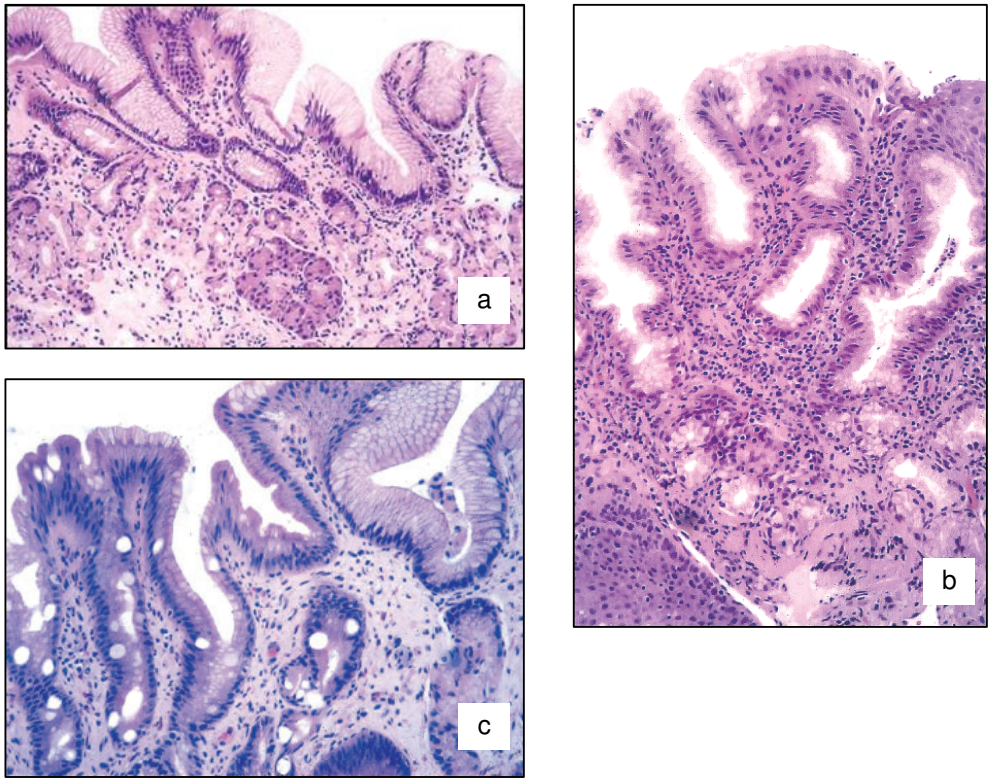


Figure 1.2: Histological subtypes of Barrett's oesophagus (a) Gastric fundic type, (b) Cardiac type, (c) Specialised intestinal metaplasia (Chandrasoma, 2005). Reproduced with kind permission of John Wiley and sons

1.3.3 The intestinal metaplasia question

The specialised columnar epithelium is often described as specialised intestinal metaplasia (SIM). The importance of SIM has been debated over the years with many authors suggesting that the diagnosis of Barrett's should only be made when this has been demonstrated. American guidelines have historically

included SIM in their definition of Barrett's whereas European guidelines have not. The most recent American College of Gastroenterology guidance recognises this inconsistency but continues to advise that SIM is required to make the diagnosis(Wang and Sampliner, 2008).

It is recognised that the yield of SIM decreases as the length of the columnar segment shortens and fewer biopsies are taken.(Harrison R et al., 2007, Wang and Sampliner, 2008) It is therefore possible that the absence of SIM in biopsies of a columnar segment reflects a sampling error, rather than a true absence of SIM in the segment. Even where SIM is demonstrated, Barrett's cannot be considered to be confirmed on the basis of histology alone as this could represent intestinal metaplasia of the cardia, a condition associated with a much lower malignant potential than Barrett's.

Until recently it was widely accepted that SIM was the epithelial type associated with the greatest, if not the only, predisposition to adenocarcinoma. Data from a Northern Ireland based cohort study (Anderson et al., 2003) indicated that only patients with SIM had a significantly increased mortality from oesophageal cancer. Recent work looking at the background epithelium in endoscopic mucosal resection specimens of small adenocarcinomas has challenged this belief (Takubo et al., 2009). In over 70% of cases, small oesophageal adenocarcinomas were surrounded by cardiac or fundic type mucosa rather than SIM.

1.4 Epidemiology of Barrett's oesophagus

The terminology used in the literature about Barrett's is particularly confusing with regards to prevalence and incidence. Prevalence is defined as the total number of existing cases as a proportion of the total population at one time. Many articles use this term but actually describe the number of new cases detected at endoscopy over a particular time period. Incidence describes the number of new cases detected over a set time period as a proportion of the population at risk. Determining the true incidence of Barrett's is virtually impossible as it is largely asymptomatic and can only be diagnosed at endoscopy. Patients attending for endoscopy are almost always a selected

group of those with upper GI symptoms and are unlikely to represent the population as a whole.

When reviewing the literature about prevalence or incidence of Barrett's oesophagus the situation is further complicated by the lack of consistent and enduring diagnostic criteria. The length of columnar lined segment required for the diagnosis and the requirement or otherwise to demonstrate specialised intestinal metaplasia are examples of inconsistencies.

Autopsy studies have attempted to determine the frequency of Barrett's in the general population. Cameron et al (1990) compared the rate of clinically diagnosed Barrett's with the rate found at unselected autopsy in a single county in the United States. The rate of clinically diagnosed Barrett's was only 23 per 100,000 residents. The estimated true rate, based on the detection of 7 cases in 733 consecutive autopsies, was more than 10 times this at 376 cases per 100,000 residents (0.4%).

More recent estimates of the prevalence of Barrett's come from endoscopic studies but the results of these vary dramatically. A Swedish study of 1000 random, unselected adults found a prevalence of 1.6% with diagnosis based on endoscopic suspicion and histological confirmation of intestinal metaplasia(Ronkainen et al., 2005). A much higher prevalence of 25% was detected in patients attending for screening sigmoidoscopy who were invited to undergo upper endoscopy at the same visit(Gerson et al., 2002). These patients were asymptomatic for reflux symptoms but were drawn from a population primarily composed of male military veterans in the United States, a group likely to be at higher than average risk due to age, smoking and drinking habits.

Whilst the incidence of oesophageal adenocarcinoma has certainly increased over the past two decades it is difficult to know whether there has been a similar upward trend for Barrett's. The number of individuals with a diagnosis of Barrett's oesophagus has definately increased but this may simply reflect changing diagnostic practice. The requirement for a 3cm segment of columnar epithelium has been abandoned, the number of endoscopies has increased and

the training of endoscopists has improved all of which may contribute to the increased frequency of diagnosis(Prach et al., 1997a). Despite this there is some evidence to suggest that there has been a real increase in cases of Barrett's oesophagus. A cohort study using a primary care database (van Soest et al., 2005) showed an increasing incidence of Barrett's between 1996 and 2003 which was independent of the number of endoscopies performed. Both the number of new diagnoses of Barrett's and the number of new cases per 1000 endoscopies in Scottish patients was shown to have increased between 1980 and 1993(Prach et al., 1997b).

1.5 Risk factors associated with Barrett's oesophagus

1.5.1 Age and gender

Barrett's is a diagnosis most often made in later life. Cameron and Lomboy (1992) reviewed the records of over 50,000 patients undergoing endoscopy over a 13 year period. Barrett's was found twice as often in men than women with a mean age at diagnosis of 63. The prevalence of Barrett's increased with age to reach a plateau by the seventh decade. Data from over 5000 patients on the UK National Barrett's Oesophagus Registry (Caygill et al., 2003) has demonstrated a male to female ratio of 1.7:1, the mean age at diagnosis being 62.0 years for males and 67.5 years for females. The Dutch cohort study (van Soest et al., 2005) reported similar findings with a mean age at diagnosis of 59.3 years for men and 65.5 years for women.

1.5.2 Race

Historically, Barrett's was thought to be a disease of white males. Recent retrospective reviews of endoscopy records have however failed to find any significant differences between rates of Barrett's in different racial groups (Bersentes et al., 1998, Fan and Snyder, 2009).

1.5.3 Gastro-oesophageal reflux disease (GORD)

Barrett's is believed to develop in response to gastro-oesophageal reflux disease and epidemiological evidence exists to support this theory. The prevalence of Barrett's has been demonstrated to be higher in patients with reflux symptoms compared to those undergoing endoscopy for other reasons

(Fan and Snyder, 2009, Johansson et al., 2007). In addition, patients with Barrett's appear to have an earlier onset and longer duration of reflux symptoms (Eisen et al., 1997). A prospective, observational study of patients undergoing endoscopy for reflux symptoms found that prevalence of Barrett's was strongly associated with the duration of reflux symptoms. Compared to patients with symptoms for less than one year, the odds ratio for Barrett's was 3.0 in those with symptoms for between 1 and 5 years, rising to 6.4 for those with symptoms dating back more than 10 years (Lieberman et al., 1997).

The association between the severity of reflux symptoms and Barrett's is less clear. Winters et al (1987), in a prospective study of 97 patients with symptomatic reflux, noted that patients with Barrett's tended to report fewer symptoms than those with oesophagitis. One reason for this may be that metaplastic Barrett's mucosa is less sensitive than squamous mucosa and the severity of symptoms therefore decreases once metaplasia is established.

1.5.4 Obesity, smoking and alcohol

Meta-analysis of epidemiological data (El-Serag, 2008) has shows that obesity is associated with a 1.5 – 2 fold increase in symptomatic GORD, the leading hypothesis being that obese individuals have greater pressure stress and anatomical disruption of the gastro-oesophageal junction. Data relating to the association between obesity and Barrett's is contradictory and it has been postulated that any increased risk associated with obesity may simply be a reflection of the increased prevalence of GORD (Cook et al., 2008).

Smoking and alcohol consumption are thought to be associated with GORD but there is limited data concerning their association with Barrett's. Several case control studies have found no association between cigarette smoking and development of Barrett's (Anderson et al., 2007, Gray et al., 1993). These are relatively small studies however and in one case concerns have been raised as to whether there was a selection bias for non-smokers in the control group. Evidence linking smoking with oesophageal adenocarcinoma is stronger, indicating a possible role in the progression, rather than the causation of Barrett's. Both studies which failed to show an association between smoking and Barrett's found a significant association with adenocarcinoma (Gray et al., 1993, Anderson et al., 2007). A further study (Gammon et al., 1997) has

reported a doubling of oesophageal adenocarcinoma risk in current and ex-smokers compared to those who had never smoked. In contrast a Swedish case control study (Lagergren et al., 2000) failed to find any significant association between smoking and oesophageal adenocarcinoma.

Alcohol is postulated to increase GORD by causing relaxation of the lower oesophageal sphincter and epidemiological evidence supports an association between alcohol and reflux disease (Locke et al., 1999). The evidence linking Barrett's oesophagus and adenocarcinoma with alcohol consumption is however, even less compelling than that for smoking. The Irish FINBAR case control study (Anderson et al., 2007) suggested an association between alcohol consumption in early adulthood and reflux disease but failed to show any association with Barrett's oesophagus or adenocarcinoma. In another study patients with uncomplicated Barrett's were significantly less likely to be drinkers than patients with severe reflux oesophagitis ($p=<0.02$)(Gray et al., 1993). In two case control studies, alcohol consumption has been found to be associated with a decreased risk of oesophageal adenocarcinoma, with odds ratios of 0.5(Lagergren et al., 2000) and 0.6.(Gammon et al., 1997)

1.6 Pathogenesis of Barrett's oesophagus

Damage to the oesophageal mucosa and an abnormal intraluminal environment during repair is required for Barrett's to develop. The exact mechanisms via which this occurs remain unclear. In particular it remains unexplained why the majority of patients with reflux do not go on to develop Barrett's.

1.6.1 Role of acid in development of Barrett's

Canine experiments provided early evidence of the acquired nature of Barrett's and the role of acid in its development. Bremner et al (1978) demonstrated that acid reflux is needed for the development of Barrett's. Mucosal defects were created in the distal oesophagus of dogs divided into three groups according to the presence, or absence, of surgically induced gastrooesophageal reflux and stimulated gastric hypersecretion. Re-epithelialisation was predominantly with squamous epithelium in dogs with an intact lower oesophageal sphincter while columnar re-epithelialisation predominated in those with reflux and gastric hypersecretion. Gillen et al (1988) refined this experiment by including an additional circumferential deficit above an intact ring of squamous mucosa. In

dogs with experimentally induced reflux disease, columnar re-epithelialisation was again seen to occur. In 2 dogs, columnar regeneration was observed above the intact ring of squamous mucosa, effectively excluding the possibility of columnar replacement by proximal migration of gastric mucosa. In the absence of reflux, re-epithelialisation was with squamous mucosa.

Further evidence for the importance of acid reflux includes the demonstration, using ambulatory monitoring, of a graded increase in reflux across the GORD spectrum (Vaezi and Richter, 1996). Patients with oesophagitis were compared with those with Barrett's oesophagus and with healthy controls. Those with complicated Barrett's had the highest amount of acid reflux, while healthy controls had the least.

Evidence regarding the role of gastric acid hypersecretion is contradictory. In a small study (Mulholland et al., 1989), both basal and stimulated acid secretion was significantly greater in Barrett's patients than controls. In a larger study however (Hirschowitz, 1996), there was no significant difference in acid and pepsin secretion in Barrett's patients and controls matched for age, sex and background gastrointestinal disease.

The mechanisms by which acid refluxate exerts its damaging effects remain unclear. For H^+ ions to cause damage they must be able to enter the cell. Oesophageal epithelial apical cell membranes are relatively impermeable to acid. It is thought that the cell cytosol becomes acidic only when the intraluminal pH is low enough to damage the intercellular junction structures allowing the H^+ ions to enter via the basolateral membranes. Once acid enters the cell it leads to cell death by necrosis, resulting in ulceration when this occurs over a large area (Carney et al., 1981).

As well as cell damage, acid appears to trigger several acute mucosal defence mechanisms including cell replication and increased blood supply (Guillem, 2005). Development of columnar metaplasia appears to be a chronic adaptive process resulting from prolonged acid exposure.

1.6.2 Role of duodenal reflux in development of Barrett's

Gillen's canine study (Gillen et al., 1988) included a bile only reflux model. Dogs in this group underwent refluxogenic surgery in the form of a cardioplasty, hiatus hernia creation and biliary diversion but were given cimetidine to suppress acid production. There were no cases of columnar re-epithelialisation in this group indicating that bile reflux alone may be insufficient to cause Barrett's. The group however included only six dogs with 3 months of reflux. Case reports of Barrett's developing after total gastrectomy, conversely, indicate that it is possible for columnar metaplasia to occur in the absence of acid reflux.(Westhoff et al., 2004, Meyer et al., 1979a)

As with acid exposure, it has been shown that there is a graded response across the GORD spectrum for both oesophageal exposure to bilirubin and fasting bile acid concentrations(Vaezi and Richter, 1996). In this study, patients with both complicated and uncomplicated Barrett's had significantly higher (89%-100%) exposure to the simultaneous damaging effect of acid and bile than those with less severe forms of oesophagitis (50-79%). This evidence supports a synergistic effect of bile and acid in the pathogenesis of Barrett's. Whilst it is important to consider the contribution of the separate components of reflux it is also important to remember that in the majority of reflux episodes, acid and duodenogastrooesophageal reflux (DGOR) occur simultaneously.

1.7 Origins of Barrett's oesophagus

Early papers on Barrett's proposed that the columnar oesophageal segment occurred as a result of proximal migration of gastric cardiac mucosa. The finding that columnar re-epithelialisation could occur above an intact ring of squamous mucosa (Gillen et al., 1988) provided evidence to the contrary and suggested that the cell of origin must lie in the oesophagus itself.

There are several postulated candidates for the cells of origin in Barrett's. The first is redifferentiation of mature squamous mucosa, a process termed 'transdifferentiation'. The second, increasingly popular theory, is conversion of pluripotent oesophageal stem cells (Fitzgerald, 2006a).

Stem cells are able to divide indefinitely to produce differentiated progeny. Under normal circumstances they divide to produce one stem cell and one transit cell resulting in tissue homeostasis. It is believed that squamous oesophageal stem cells reside in the basal compartment of the squamous epithelium. Various mechanisms for the development of Barrett's from stem cells have been proposed (Jankowski et al., 2000).

The *de novo* metaplasia theory suggests that damage may occur to exposed stem cells in inflamed mucosa. This damage converts the squamous stem cells to Barrett's stem cells which repopulate the oesophagus with columnar mucosa. The duct-cell metaplasia theory proposes that when squamous mucosa is damaged, stem cells in the glandular neck region of oesophageal submucosal ducts are able to colonise the oesophageal mucosal layer. Less well recognised theories suggest that Barrett's could originate from circulating bone marrow cells (Sarosi et al., 2008), from a residual population of embryonic cells at the gastro-oesophagaeal junction (Wang et al., 2011) or from the oesophageal stroma (Chang et al., 2007).

The mechanism by which metaplasia spreads throughout the oesophagus is also poorly understood. Recent work has sought to determine the clonality of Barrett's mucosa i.e. whether it is mono or polyclonal. It was initially believed that the phenotypic change occurred as a result of a single stem cell mutation with a selective advantage enabling it to expand to fill an entire Barrett's segment. Evidence for this came from work showing apparently clonal lesions of both p16 and p53 genes throughout long segments of Barrett's (Wong et al., 2001, Galipeau et al., 1999). Recent work by Leedham et al. (2008) has refuted this and suggested that Barrett's arises from multiple independent clones. This group used laser capture microdissection to enable analysis of Barrett's segments at a much higher resolution than had previously been possible.

1.8 Barrett's and oesophageal adenocarcinoma

1.8.1 Association of Barrett's and oesophageal adenocarcinoma

The importance of Barrett's oesophagus lies in the association with oesophageal adenocarcinoma, a condition for which it is the major risk factor.

The incidence of oesophageal adenocarcinoma has risen dramatically since the 1970s and the prognosis remains dismal(Devesa et al., 1998, Bollschweiler et al., 2001).

The first case report of adenocarcinoma arising within a columnar lined oesophagus (Morson and Belcher, 1952) was published only two years after Barrett's original paper. By the 1970s it was becoming recognised that the condition had significant malignant potential. Naef (1975) reported a series of 12 adenocarcinomas arising from a cohort of 140 patients with extensive columnar metaplasia. Haggitt et al (1978) reported that 12 of 14 cases of primary oesophageal adenocarcinoma occurred on a background of columnar lined oesophagus. In 10 cases, the columnar epithelium adjacent to the invasive cancer showed a spectrum of abnormalities ranging from dysplasia to carcinoma *in situ*, supporting the idea of progression from metaplasia to dysplasia and carcinoma.

It is now believed that most, if not all, cases of oesophageal adenocarcinomas arise on a background of Barrett's oesophagus, albeit previously unrecognised in the majority of cases. A systematic review (Dulai et al., 2002) found that only 5% of patients undergoing resection for oesophageal adenocarcinoma had a diagnosis of Barrett's preceding their cancer diagnosis. The earliest literature analysed by this group dated back to the 1960s. Whilst one might expect this figure to be higher for today's patients, given the increased use of endoscopy and awareness of Barrett's among clinicians, it remains the case that the vast majority of patients diagnosed with adenocarcinoma have no prior diagnosis of Barrett's. Where Barrett's is not identified at the time of diagnosis it has been hypothesised that this is a result of tumour overgrowth beyond the area of pre-existing Barrett's (Chandrasoma et al., 2007). This view is supported by the finding that Barrett's can be 'unmasked' following neo-adjuvant chemotherapy. In a retrospective study of 79 patients the rate of identifiable co-existing Barrett's rose from 75% to 97% following chemotherapy (Theisen et al., 2002).

1.8.2 Magnitude of the risk

Perhaps surprisingly considering the well established association between Barrett's and oesophageal adenocarcinoma, the actual risk of developing

cancer in a Barrett's oesophagus is unclear. Estimates range from 0.1% to nearly 3% per patient year with a recent meta-analysis of European and American literature quoting an overall risk of 7:1000 years patient follow up (Thomas et al., 2007). It is important to recognise that differing definitions of Barrett's have been used and this in part must explain some of the variation in quoted risks. Short segment Barrett's for instance was not included in most early series and this appears to be associated with a lower risk of malignant progression (Thomas et al., 2007).

A publication bias, with significant over-representation of the cancer risk in Barrett's oesophagus has been suggested. Shaheen et al (2000) found a strong inverse relationship between study size and reported cancer risk, with larger studies reporting much lower cancer risks. A recent population based cohort study from Denmark (Hvid-Jensen et al., 2011) included data on over 11000 patients from a comprehensive national registry, median follow up was 5.2 years. The incidence of adenocarcinoma was highest in the first year after diagnosis suggesting that prevalent cancers were initially missed. Beyond this the annual risk of adenocarcinoma fell to 0.12%, much higher than the risk for the background Danish population (Relative risk 11.3) but much lower than the figures usually quoted.

Whilst Barrett's is clearly associated with a significantly increased relative risk of oesophageal adenocarcinoma it is important to retain some perspective on such matters. Oesophageal cancer remains a comparatively rare condition with only 8161 new cases diagnosed in the UK in 2009 (Cancer Research UK Statistics, 2012), the last year for which figures are available. The vast majority of patients with Barrett's oesophagus never develop oesophageal cancer. A retrospective cohort study of all patients identified with Barrett's oesophagus in Northern Ireland between 1993 and 1999 found that overall mortality for such patients was not raised and that oesophageal cancer remained an uncommon cause of death (Anderson et al., 2003). During 7413 person years of follow up there were 253 deaths, only 12 of which were from oesophageal cancer. This figure was, however, significantly higher than expected, equating to a standardised mortality rate of 518. When analysed by subgroup it was found that only

patients with specialised intestinal metaplasia had a significantly increased oesophageal cancer mortality rate.

1.8.3 The metaplasia – dysplasia – carcinoma sequence

It is believed that adenocarcinoma develops from Barrett's metaplasia via a stepwise progression through low and high grade dysplasia (Jankowski et al., 1999). For individuals who progress, it has been suggested that genetic mutations accumulate over time. These may confer a selective advantage on the abnormal cell allowing it to clonally expand, eventually forming an invasive cancer (Fitzgerald, 2006a).

Metaplasia is defined as the transformation of one type of mature differentiated cell into another fully differentiated cell type. Metaplasia often represents an adaptive response of a tissue to environmental stress and it is thought that the metaplastic epithelium is better able to withstand the adverse environmental changes (Underwood, 1998). In the case of Barrett's oesophagus it is thought that the columnar epithelium is more resistant to the damaging effects of reflux.

1.8.4 The natural history of progression

Epidemiological evidence suggests that Barrett's develops rapidly to its full length with little subsequent change. Review of endoscopy records of 21 patients followed up for a mean of 7.3 years showed a mean initial length of 8.29cm and a mean final length of 8.33cm (Cameron and Lomboy, 1992). Metaplasia does not necessarily progress to dysplasia but in the presence of ongoing environmental insult it has the potential to do so.

1.8.5 Dysplasia in Barrett's oesophagus

Dysplasia is defined as an unequivocal neoplastic alteration of epithelium which has the potential to progress to invasive malignancy but remains confined within the basement membrane of the epithelium within which it arose (Riddell et al., 1983). It is the only marker of increased risk in Barrett's which is widely used in clinical practice and is diagnosed on the basis of both cytological and architectural abnormalities. Hameeteman and collegues (Hameeteman et al., 1989) demonstrated an increase in both the frequency and severity of dysplasia in a cohort of 50 Barrett's patients followed, prospectively for a mean of 5

years.(Hameeteman et al., 1989) British Society of Gastroenterology guidelines (2005) recommend that Barrett's biopsies are histologically reported according to a six-point scale of neoplastic change.

1. Negative for dysplasia
2. Indefinite for dysplasia
3. Low grade dysplasia
4. High grade dysplasia
5. Intramucosal carcinoma
6. Invasive adenocarcinoma

Dysplasia within a Barrett's segment occurs in patches which may be unifocal or multifocal. There is great potential for sampling error and dysplasia can be missed if insufficient biopsies are taken. It is therefore recommended that when assessing a Barrett's segment for dysplasia, quadrantic biopsies should be taken every 2cm and from any macroscopic lesion within the segment.

1.8.6 Low Grade Dysplasia (LGD)

Low grade dysplasia can be difficult to distinguish from inflammatory changes and diagnosis should be confirmed with repeat biopsies following PPI therapy. In one series (Conio et al., 2003) 75% of cases of LGD were not confirmed on repeat biopsy. The natural history of LGD is poorly understood. The majority of patients do not appear to progress beyond this stage and regression has been reported in over 60% of cases (Weston et al., 2001, Skacel et al., 2000). A subgroup of patients with LGD do however, progress along the metaplasia-dysplasia-carcinoma sequence. Weston et al. (2001) detected progression in 10% of a cohort of 48 patients followed for a mean of 41 months. Where there is agreement as to the diagnosis of LGD the risk of progression understandably appears to be higher. In one study, where there was a consensus of opinion between 3 pathologists, 4 out of 5 patients progressed.(Skacel et al., 2000) The time taken to progress from onset of metaplasia is impossible to determine accurately as metaplasia may be present for many years prior to diagnosis. However in a group of seven patients who progressed sequentially from metaplasia to cancer, the median time from diagnosis of Barrett's to the development of LGD was only 24 months (Theisen et al., 2004).

1.8.7 High Grade Dysplasia (HGD)

The behaviour of high grade dysplasia is also difficult to predict. It can progress to adenocarcinoma rapidly, slowly or not at all. Schnell and colleagues (2001) reported 12 cases of adenocarcinoma in a series of 75 patients with HGD followed up for a mean of 7 years from the time of diagnosis of Barrett's (range 1.5-14years). Diffuse HGD appears to represent a higher risk than focal HGD. Cancer rates after 3 years follow up for HGD were 56% for those with diffuse HGD vs 14% for those with focal HGD in one retrospective cohort study (p=0.002) (Buttar et al., 2001). In the series of patients with sequential progression from metaplasia to adenocarcinoma, median time from the diagnosis of Barrett's to the diagnosis of HGD was 33 months. The median time to the diagnosis of cancer was only 3 months more at 36 months(Theisen et al., 2004).

Regression of HGD has been reported (Weston et al., 2000). In this study regression to LGD was observed in 2 out of 15 surveyed patients and regression to no dysplasia in 5 of the 15. This small study followed patients with unifocal high grade dysplasia for a mean period of 36.8 months. The original diagnosis was confirmed by a second blinded pathologist, reducing the chance that apparent regression reflected inter-observer variation in diagnosis but a second potential source of error is less completely addressed. A failure to resample the original area of dysplasia could clearly result in a false finding of regression. This study actually reported 8 cases of progression in the 15 patient cohort, 4 to invasive adenocarcinoma, 3 to possible intramucosal adenocarcinoma and 1 to multifocal HGD and it must therefore be regarded overall as providing supportive evidence for the malignant potential of HGD.

There are no other studies which clearly describe spontaneous regression of HGD. The presence of HGD has widely been viewed as an indication for oesophagectomy, or endoscopic treatment in more recent years and evidence regarding the natural history of untreated oesophageal HGD is consequently very limited.

In contrast to the suggestion that HGD may regress, there is reasonable evidence that this finding may be associated with more advanced disease.

Several surgical series of patients undergoing resection for HGD report rates of occult adenocarcinoma of up to 40% (Heitmiller et al., 1996, Falk et al., 1999, Reed et al., 2005).

1.9 Clinical challenges in Barrett's oesophagus and oesophageal adenocarcinoma

The incidence of oesophageal adenocarcinoma continues to increase yet there has been relatively little improvement in prognosis over several decades. Neo-adjuvant chemotherapy has been shown to confer a survival advantage and surgical outcomes have significantly improved with operative mortality now consistently less than 5% in specialist centres.(Medical Research Council, 2002, van Lanschot et al., 2001) (National Oesophago-gastric Cancer Audit, 2010) Despite these advances the majority of patients present at a stage when curative treatment is not possible and overall 5 year survival remains poor at only 13% (Office for National Statistics, 2012). It seems unlikely that there will be significant progress in the treatment of advanced oesophageal adenocarcinoma in the foreseeable future and there is therefore an impetus to address the issues of cancer prevention and early detection.

Endoscopic screening for Barrett's oesophagus to detect patients at elevated risk of oesophageal carcinoma is not routinely recommended and is unlikely to ever be cost-effective (British Society of Gastroenterology, 2005, (Wang and Sampliner, 2008)). Given the recent studies suggesting that the absolute risk in Barrett's oesophagus is much lower than previously believed, it is likely to be increasingly difficult to justify widespread surveillance of patients with known Barrett's oesophagus in a healthcare environment with increasing pressure on resources. Developments in endoscopic technologies such as narrow band imaging and chromoendoscopy may improve detection of areas of dysplasia and early adenocarcinoma within Barrett's oesophagus and ensure that surveillance and index endoscopy is as accurate as possible (Spechler et al., 2011).

The greatest advances in reducing the disease burden of oesophageal adenocarcinoma are likely to come as a result of better understanding of the development and progression of the disease. There has been interest in the

use of drugs to prevent the malignant progression of Barrett's oesophagus, so called chemoprevention and a large randomised trial seeks to evaluate the efficacy of aspirin (Das et al., 2009). There has also been significant interest in the identification and use of biomarkers to risk stratify patients with reflux disease and Barrett's oesophagus and allow screening and surveillance to be more effectively targeted at high risk individuals (Kadri SR et al., 2010, Spechler et al., 2011). These techniques are yet to become routine but clearly have the potential to affect practice. An improved understanding of the pathogenesis of both Barrett's oesophagus and oesophageal adenocarcinoma could clearly also open the door to new molecular treatment modalities.

1.10 Models of Barrett's Oesophagus

Evaluation of therapeutic candidates both for the prevention of Barrett's in those with reflux and the prevention of malignant progression in those with Barrett's require expensive long-term randomised trials. Insight into the timescale over which reflux related damage occurs would require endoscopic surveillance of large numbers of healthy individuals over many years which is neither ethically acceptable nor financially viable. Models are therefore required in which to study the pathogenesis of Barrett's oesophagus and to fast-track evaluation of potential therapeutic interventions.

The aims of a Barrett's oesophagus model are:

1. To allow observation and investigation of the earliest stages of development of Barrett's
2. To allow the study of malignant progression over an accelerated timescale
3. To allow potential therapeutic interventions to be assessed safely and over an acceptable timescale
4. To allow identification of genetic or biological factors associated with the development or progression of Barrett's

Requirements of a good Barrett's oesophagus model include the following:

1. Spontaneous metaplasia should occur in the model species
2. Model species should be genetically similar to man
3. Model should include a lifelike model of reflux

4. Model should represent the complex confounding factors in humans eg. Ageing, lifestyle factors and genetic heterogeneity

There is no perfect model of Barrett's oesophagus but *in vitro, ex vivo*, animal and human models all have their relative strengths and weaknesses.

In vitro techniques using cell lines allow straightforward experimental data to be obtained. Various factors can be manipulated or introduced and the effects on cell viability, apoptosis and biomarker expression observed. The drawback with this model is that cells grown *in vitro* are not subject to normal physiological conditions and their growth behaviour can be heavily influenced by the immortalisation procedures used in their creation (di Pietro M et al., 2008).

Ex vivo models using biopsy samples in culture allow the study of oesophageal cells in their normal tissue arrangement but these can only be kept alive for a short period of time. This model can therefore not be used to address complex temporal issues, it provides no opportunity to assess the long term effects of ongoing damage or to assess if potential chemotherapeutic agents are effective (di Pietro M et al., 2008).

Animal models of Barrett's have been used for many years; the first evidence of the acquired nature of columnar metaplasia came from a canine model. Other widely used models utilise rats and mice. Clearly there are genetic differences between animal models and the human situation they aim to represent but other differences are equally important (Attwood et al., 2008). Rodents do not have spontaneous reflux and require surgical intervention to induce this. This tends to produce supra-physiological levels of reflux and the surgical insult also has immunomodulatory effects. Depending on the species there are also concerns about animal models with regards to the absence of submucosal oesophageal glands. These glands are thought to be important in the development of human Barrett's oesophagus and have been proposed as a potential site for a Barrett's oesophagus stem cell (Jankowski et al., 2000). If these glands are absent it may be that columnar metaplasia in these species develops via a different mechanism to that in humans. In addition most animals do not naturally

develop oesophageal adenocarcinoma, a further divergence from the disease spectrum observed in humans.

The recognised deficiencies of these models have led to interest in the human model of Barrett's oesophagus which is observed in patients following subtotal oesophagectomy and reconstruction with a gastric conduit. During this procedure the gastro-oesophageal junction and distal oesophagus are excised and the stomach is anastomosed to the proximal oesophagus to re-establish intestinal continuity. A short segment of residual oesophagus remains in situ and is commonly referred to as the oesophageal remnant.

1.11. Barrett's in the post oesophagectomy patient – 'Neo-Barrett's'

The development of columnar metaplasia in the remnant native oesophagus following surgery has been described by several groups in both paediatric and adult populations. (Borgnon et al., 2004, Lindahl et al., 1990, Hamza et al., 2003, O'Riordan et al., 2004, Oberg et al., 2002, Lord et al., 2004, Dresner et al., 2003, Franchimont et al., 2003). In adults it has been shown to occur following surgery for both adeno and squamous carcinoma with an incidence of up to 50%.(O'Riordan et al., 2004) This group of patients is prone to profound reflux providing a potential mechanism for the development of Barrett's.

1.11.1 Reflux post-oesophagectomy

Reflux of both gastric and duodenal contents is very common following oesophagectomy with a gastric conduit reconstruction. The majority of patients report some symptoms consistent with reflux (Aly and Jamieson). Dresner et al. (2003) demonstrated abnormal amounts of oesophageal exposure to both acid and bilirubin over a 24 hour period in over 80% of post operative patients and similar findings were reported by Oberg (Oberg et al., 2002).

The reasons for this high prevalence of reflux relate to the disruption of the normal anatomical anti-reflux mechanisms. The lower oesophageal sphincter, angle of His and diaphragmatic sling are all resected or disrupted. In addition reflux is promoted by the position of the gastric tube between the positive pressure environment of the abdominal cavity and the negative pressure of the thoracic cavity.

1.11.2 The importance of post-oesophagectomy patients as a human model for the development of Barrett's

Post-oesophagectomy patients provide an exceptionally useful model in which to study the early events in the development of Barrett's oesophagus. Examination of the original resection specimen allows confirmation of normal squamous epithelium above the anastomosis at the time of surgery and enables a timescale for the development of metaplasia to be determined.

Another important feature of this group is the relative ease with which the precise location of biopsies can be identified. The surgical anastomosis is easily identified at endoscopy and any biopsies from above this are clearly oesophageal. Any histological finding in supra-anastomotic biopsies other than squamous mucosa is metaplastic. The difficulties normally associated with accurately identifying the gastro-oesophageal junction are therefore eliminated.

Where the resection specimens have been retained, examination of the proximal margin and comparison with the neo-Barrett's material from the same patient provides a unique opportunity to study the molecular and genetic characteristics associated with the phenotypic change.

1.11.3 Characteristics of post-oesophagectomy neo-Barretts

The endoscopic appearance of neo-Barrett's tissue is essentially the same as that of sporadic Barrett's. The mucosa has a darker appearance than that of the adjacent squamous tissue. Histological assessment of samples taken from segments of neo-Barretts has shown both cardiac type mucosa and specialised intestinal metaplasia. These neo-Barrett's epithelia have an identical histological appearance to their sporadic Barrett's equivalents.

Chapter 2. Comparative Literature Review

Clinical follow up for patients who have undergone subtotal oesophagectomy is highly variable. Many units do not routinely endoscope patients and those that do rarely conform to a standard protocol. In many cases endoscopy is undertaken to investigate symptoms and to exclude the presence of local recurrence of adenocarcinoma. The endoscopist may not undertake a thorough evaluation of the oesophageal remnant and biopsies may be taken only where there is a suspicion of significant pathology. Columnar metaplasia or 'neo-Barrett's may go unrecognised by inexperienced endoscopists, particularly if they are unfamiliar with the post surgical anatomy. These factors, along with the relatively small numbers of patients who undergo oesophagectomy go some way towards explaining the paucity of evidence available on neo-Barrett's oesophagus.

2.1 Aim of the literature review

The aim of this review was to summarise the existing literature on the incidence and characteristics of neo-Barrett's metaplasia occurring following oesophagectomy.

2.2 Search Strategy

Whilst this thesis uses the term 'neo-Barrett's' to describe columnar metaplasia occurring in the oesophageal remnant following subtotal oesophagectomy this is not a widely recognised term. Searches of the major scientific databases for this term return no relevant papers.

The search strategy outlined in table 2.1 was used to search both Medline 1946-2012 and Embase 1980-2012. The search strategy was adapted for the Web of Science and Scopus databases (figures 2.1 and 2.1). The bibliographies of relevant papers were hand searched for other relevant cited articles.

#	Search term
1	Barrett Esophagus/
2	Columnar metaplasia.mp.
3	1 or 2
4	Esophagectomy/
5	Esophagoplasty/
6	Surgical anastomosis/
7	4, 5 or 6
8	esophageal remnant.mp.
9	oesophageal remnant.mp.
10	8 or 9
11	3 or 7
12	10 or 11

Table 2.1: Literature review search strategy for Medline and Embase

```
TS=("barrett* *esophagus" or "columnar metaplasia") AND
TS=(*esophagectomy or *esophagoplasty or "surgical anastomosis")
AND TS=(*esophageal remnant)
```

Figure 2.1: Search strategy for Web of Science database

```
(TITLE-ABS-KEY("barrett* *esophagus" OR "columnar metaplasia") AND
TITLE-ABS-KEY(*esophagectomy OR *esophagoplasty OR "surgical
anastomosis") AND TITLE-ABS-KEY("*esophageal remnant"))
```

Figure 2.2: Search strategy for Scopus database

2.3 Scope of the literature review

The topics of interest covered by the literature review are listed below:

Prevalence of neo-Barrett's in the adult surgical population
Prevalence of neo-Barrett's in the paediatric surgical population
Timescale for the development of neo-Barrett's
Pre-disposing factors for the development of neo-Barrett's
Malignant progression in neo-Barrett's
Molecular marker expression in neo-Barrett's

2.4 Methods

The studies identified in the search will be assessed for the quality of their data according to the criteria set out in table 2.2.

Quality criteria for literature review
Number of patients
Prospective endoscopic evaluation
Histological corroboration of neo-Barrett's
Histological exclusion of residual disease at time of surgery
Inclusion criteria eg. Routine follow up of all patients, investigation of research volunteers or investigation of symptomatic patients

Table 2.2: Quality criteria for literature review

2.5 Prevalence of neo-Barrett's in the adult surgical population

Twelve studies were identified which evaluated the prevalence of neo-Barrett's, these are summarised in table 2.3 below. The quality of these studies varies, all are from single centres. All studies report cases of neo-Barrett's during the follow up period. The studies will be discussed in chronological order.

Öberg and colleagues (2002) published the first study which sought to evaluate the prevalence of columnar metaplasia in the remnant oesophagus following oesophagectomy and gastric tube reconstruction. All 60 surviving patients who had undergone surgery in the unit were invited to participate. Thirty two patients underwent prospective endoscopic evaluation and 15 cases of columnar metaplasia were detected (47%). The strengths of this study include the histological confirmation of squamous mucosa at the resection margin at the

time of surgery and the routine biopsy of areas of apparent oesophagitis to exclude the presence of metaplasia. One potential weakness of this study is the inclusion of only a subset of the group of survivors as whole. The authors recognise this fact and that this may give rise to a degree of bias towards patients more symptomatic for reflux disease but they state that the major reason for decline of the study invitation was old age or medical co-morbidities rather than the absence of symptoms.

A similar study was published by Dresner et al (2003) the following year. Again all surviving patients from the authors unit were invited to participate and 20 of 51 agreed to do so. Nineteen cases of columnar metaplasia were identified (48%) and of these 9 (23%) demonstrated specialised intestinal metaplasia. As in the Öberg study, endoscopy was prospective, by an endoscopist with a specialist research interest and there was routine exclusion of residual Barrett's and histological confirmation of neo-Barrett's. Again there is a potential selection bias towards patients who had more symptomatic reflux disease. Both of these studies included 24 hour acid and bilirubin monitoring in addition to endoscopic evaluation and it is possible that patients who were asymptomatic were less willing to undergo this degree of monitoring.

Also published in that year, a study by Franchimont and colleagues (2003), reported a 13.5% prevalence rate of neo-Barrett's metaplasia amongst a cohort of 66 patients who had undergone subtotal oesophagectomy in a single surgical unit. Unlike the previous two studies this one was based on a retrospective review of medical records. The patients were drawn from a group of 87 patients with 21 excluded due to missing data, no upper gastrointestinal endoscopy or residual Barrett's oesophagus (one case). The authors do not state the indication for post operative endoscopy employed in the unit and it is therefore difficult to assess if there might be a selection bias in the included patients. It is not clear whether the endoscopist specifically assessed for the presence of neo-Barrett's. The strength of this study is the routine use of biopsy sampling but the location of these biopsies was not standardised and they are simply described as having been taken from 'around the oesophagogastric anastomosis'. Clearly biopsies taken from below the surgical anastomosis would be of no use in confirming the presence of neo-Barrett's.

In 2004 a further four studies were published which assessed the prevalence of neo-Barrett's. The largest of these came from the Dublin group (O'Riordan et al., 2004). This was a prospective case series of 48 patients. This included a consecutive series of patients invited to participate with no refusals recorded. Strengths of this study include the prospective evaluation by one of two experienced surgical endoscopists and the routine biopsies from 1-2cm above the anastomosis. Additional biopsies were taken from suspected areas of neo-Barrett's and oesophagitis. All resection specimens were reviewed to exclude residual Barrett's oesophagus. Interestingly this study reports 10 patients who had histological evidence of columnar metaplasia but no associated endoscopically visible metaplasia. No explanation for this finding is given by the authors and this incidence of metaplasia unrecognised by endoscopists is not reported elsewhere.

The only other prospective study published in 2004 is much smaller and included only 14 patients (Peitz et al., 2004b). These appear to be a consecutive series of patients undergoing endoscopy for a variety of clinical indications rather than purely in a research setting. Again this study benefits from review of histology to exclude residual Barrett's oesophagus and the authors describe being able to clearly identify the anastomosis during endoscopy. In one case the patient did not undergo biopsy of an endscopically visible area of metaplasia due to concerns about bleeding. The main focus of this study was the anastomosis itself rather than the remnant oesophagus above this. In all cases the anastomosis is described as being covered by columnar epithelium, a finding confirmed histologically in the 13 patients suitable for biopsy with 10 cases of cardiac mucosa and 3 of oxyntic mucosa. There were 10 cases of endoscopically visible columnar metaplasia above the anastomosis and this was confirmed histologically in 9 cases.

Two further studies were published in 2004, both of which were retrospective case series based on existing endoscopy records. The larger of these (Wolfsen et al., 2004) involved 36 patients who had undergone post operative endoscopy and biopsy who were identified from a series of 45 patients who had undergone subtotal oesophagectomy. Surgical specimens were reviewed to ensure that the proximal margin was completely free of Barrett's metaplasia, dysplasia or

carcinoma. Indications for endoscopy are not stated and this series included a high proportion of patients who required dilatation of an anastomotic stricture during endoscopy (16/36, 44%). The concern here is that examination of the oesophageal remnant and biopsy regime may have been less meticulous if the primary aim was therapeutic intervention. Eight cases of neo-Barrett's are reported (18%) at a median time from surgery of 42 months, the follow up period for those without neo-Barrett's is not reported.

The second retrospective case series published in 2004 was also from the United States (Lord et al., 2004). The authors of this study reviewed the records of 100 patients who had undergone subtotal oesophagectomy and gastric tube reconstruction and identified 20 who had subsequent endoscopic biopsy of the oesophageal remnant. In 10 cases columnar metaplasia was identified in the oesophageal remnant. Endoscopic follow up was not routine in this unit and therefore all patients were symptomatic at the time of investigation for regurgitation, dysphagia, chest pain or weight loss, giving rise to potential selection bias as seen in many such studies. As described above, patients were only included if biopsies were available. The authors state that 'biopsies were performed to conduct studies such as the present one but what is not clear is whether all patients undergoing post operative endoscopy underwent biopsy sampling. This makes the true denominator for this series very difficult to determine, if biopsies were only taken when there was a suspicion of mucosal abnormality the true denominator might be much larger and the prevalence of neo-Barrett's much lower.

In 2007 a retrospective case series was published based on the records of 613 patients who had undergone oesophagectomy and gastric tube reconstruction over a 10 year period (Bax et al., 2007). The stated aims of this study were to determine whether gastric-type mucosa in the oesophagus is a precursor stage of intestinal metaplasia but the prevalence of neo-Barrett's is also evaluated. The authors identified 45 patients who had undergone endoscopic evaluation 6 months or more following surgery. There were 18 cases of neo-Barretts giving a stated prevalence of 40%. All of these had biopsy samples available for confirmation. There were 7 cases of neo-Barrett's with specialised intestinal metaplasia and no cases of dysplasia. This study has a number of

weaknesses. There is no evidence that residual Barrett's oesophagus at the time of surgery was excluded. Patients who underwent endoscopy did so primarily for dilatation of strictures or because there was a suspicion of recurrent malignancy. The level of experience of the endoscopist is not recorded and there is no record of whether the examination included any specific assessment for the presence of neo-Barrett's. There is no record of the location of biopsies and the macroscopic findings at the time of endoscopy are poorly recorded.

One of the largest series to assess the prevalence of neo-Barrett's following oesophagectomy was published in 2008 (da Rocha et al., 2008) Prevalence rates of 11% at 5 years, 30% at 5-10 years and 58% over 10 years are reported. This study from Brazil involved a very different patient group to the others identified by this literature search. All patients underwent surgery for advanced achalasia secondary to Chaga's disease. In addition to its size (101 patients), this study benefits from a number of strengths. Endoscopic follow up every two years with multiple biopsies was routine in these patients and the presence or absence of oesophagitis or columnar metaplasia in the oesophageal remnant was apparently routinely recorded. The study also benefits from the longest follow up periods of any such study. Patients were younger than those included in the European and North American studies and the indication for surgery was benign in all cases. The study is not without some weaknesses however. There is no indication that there was exclusion of Barrett's oesophagus at the time of surgery although clearly this is less likely given the indication for surgery. The series includes patients with follow up periods of up to 40 years. Given that this precedes the first description of neo-Barrett's and modern high definition endoscopes there might be some concerns as to whether the evaluation patients early in this series is as reliable as that for later patients.

A year later in 2009 the largest prospective study of metaplasia in the oesophageal remnant was published (D'journo et al., 2009). Eighty four patients underwent endoscopic evaluation. There were 21 cases of endoscopically visible columnar metaplasia (25%) and 42 cases of histologically evident columnar metaplasia (50%). The reason for the significantly higher

prevalence of metaplasia is not adequately explained and this degree of discrepancy is not reported in other series. The authors do report significant numbers of patients with endoscopic evidence of ulceration and erosions and whether these actually represent unrecognised columnar metaplasia is unclear. The indication for endoscopy for patients in this study is not clearly stated. The majority of patients (64%) admitted to reflux symptoms when questioned but it is unclear if patients were included on the basis of their symptoms or if they were research volunteers. Despite these issues this study has a number of high quality features. The authors describe a meticulous technique for examination and biopsy sampling of the oesophageal remnant. Surgical resection specimens were reviewed to exclude the presence of residual Barrett's oesophagus. The examining pathologist was blinded to the endoscopic findings and there was subsequent correlation between endoscopic and histological findings.

The final study identified by the literature search was published in 2010 (Nishimura et al., 2010). This study predominantly considered reflux oesophagitis in the oesophageal remnant but there is some data on the prevalence of neo-Barrett's. Data was available for 100 patients at one year and a 14% prevalence of neo-Barrett's is quoted. Fifty eight patients had two year follow-up data available and 23 cases of neo-Barrett's were identified (40%). This study is one of the largest but is of relatively poor quality. It is based on retrospective review of endoscopy records. The authors identified 289 patients who had undergone surgery, only 100 patients are included in the study and no inclusion criteria are stated. The resection margins were apparently not reviewed to exclude the presence of residual Barrett's oesophagus. Of the 100 patients, 98 are reported to have undergone surgery for cancer. The study originates from Japan and one would expect the vast majority of these to be squamous cell cancers where co-existing and residual Barrett's is extremely unlikely but there no evidence is presented to confirm this. The authors describe columnar lined oesophagus in these patients but this is not defined and it is unclear from the paper whether any histological confirmation of this finding was available.

The most recent paper identified by this literature review was published sometime after the present study began (Tsiouris et al., 2011). This was a retrospective study of endoscopy records and included patients who had undergone surgery using a variety of techniques, all of which involved resection of the gastro-oesophageal junction. The authors identified 151 patients who had undergone endoscopy at least one year after surgery from a total surgical cohort of 179 patients. The indications for endoscopy are not stated. The aim of this study was to compare the outcomes following standard surgical techniques with a new novel technique developed by the authors for concomitant fundoplication. Thirteen cases of Barrett's in the oesophageal remnant were identified but in one case there was evidence of residual Barrett's oesophagus when the original resection pathology was reviewed. This study used an American definition of Barrett's oesophagus and therefore only included patients with specialised intestinal metaplasia in the definition. There is no data presented on whether other types of metaplasia were observed or the incidence of endoscopic Barrett's oesophagus.

In summary there is clear evidence that neo-Barrett's occurs in a significant proportion of patients following subtotal oesophagectomy and reconstruction with a gastric conduit. Columnar epithelium both with and without specialised intestinal metaplasia occurs. The overall prevalence from the identified studies is 38% but the size and quality of the studies is somewhat limited and the largest study had to be excluded from this calculation as it did not report the incidence of non-intestinalised columnar epithelium. The overall prevalence of specialised intestinal metaplasia is 17%. This phenomenon can occur in patients who have no previous history of Barrett's oesophagus or oesophageal adenocarcinoma.

Author, publication, year, country	No. of patients	Follow-up period mths Median (range)	Study type	Inclusion criteria	Histological corroboration of neo-Barrett's	Residual Barrett's effectively excluded	Incidence of columnar metaplasia	Incidence of specialised intestinal metaplasia
Öberg S, et al. Ann Surg 2002 Sweden	32	58 (36-125)	Prospective	Research volunteers	Yes	Yes	15 (47%)	3 (9%)
Dresner SM, et al. Br J Surg 2003 UK(Dresner et al., 2003)	40	38 (13-118)	Prospective	Research volunteers	Yes	Yes	19 (48%)	9 (23%)
Franchimont D, et al. Endoscopy 2003 Belgium	66	16 (1-39)	Retrospective	Not stated	Yes	Yes	15 (23%)	9 (13.5%)
O'Riordan JM, et al Am J Gastro 2004 Ireland	48	26 (12-67)	Prospective	Consecutive patients in a research setting	Yes	Yes	24 (50%)	13 (27%)
Peitz U, et al Gastrointest Endosc 2004 Germany	14	27 (3-88)	Prospective	Consecutive patients with clinical indication for endoscopy	Yes (in 13 of 14 patients)	Yes	13 (93%)	3 (21%)
Wolfsen HC, et al BMC Gastro 2004 USA	36	42 (7-90)*	Retrospective	Not stated	Yes	Yes	8 (22%)	8 (22%)
Lord RVN, et al. Surgery 2004 USA	20	36 (9-504)*	Retrospective	Clinical indication for endoscopy and available tissue	Yes	Yes	10 (50%)	4 (20%) 1 Intramucosal cancer 42yrs post-op

Bax D, et al. J Clin Gastroenterol 2007 Netherlands	45	59 (6-148)	Retrospective	Patients with clinical indication for endoscopy	Yes	No	18 (40%)	7 (16%)
Da Rocha JFM, el al. Ann. Surg. Oncol. 2008 Brazil	101	Mean 126 +/- 106	Retrospective	All patients	Yes	No	36 (36%)	23 (23%)
D'Journo XB, et al. Ann Surg 2009 Canada	84	35 (1-295)	Prospective	Not stated	Yes Significant discrepancy between endoscopic findings and histology	Yes	42 (50%)	17 (20%)
Nishimura K, et al. Dis Esoph 2010 Japan	100 (subgroup of 58 patients)	12 (24)	Retrospective	Not stated	No	No	14 (14%) 23 (40%)	Not assessed
Tsiouris A, et al. World J Surg 2011 USA	151	Average not stated (6mths – 10yrs)	Retrospective	Not stated	Yes	Yes	Not assessed	12 (8%)
Totals	737						223 (38% of those assessed)	108 (17% of those assessed)

Table 2.3: Summary of published literature relating to post-oesophagectomy 'neo-Barrett's'

2.6 Prevalence of neo-Barrett's in the paediatric surgical population

Oesophageal surgery in children is almost exclusively performed for benign disease. In contrast to those undergoing surgery as adults, primarily for malignant disease, paediatric patients are expected to live for many decades post-operatively. Given that these children have the same anatomical predisposing factors for reflux as adult patients, the oesophageal remnant can be expected to be exposed to prolonged, high levels of duodenogastro-oesophageal reflux. The concern must be that these individuals are at high risk of developing neo-Barrett's and are likely to live long enough after their operation to progress along the metaplasia-dysplasia-carcinoma sequence. Five studies were identified which assessed the prevalence of neo-Barrett's following paediatric surgery

The first of these studies was published in 1990 by the Helsinki group (Lindahl et al., 1990). The authors identified 18 long-term survivors (> 2 years) following gastric tube reconstruction for a variety of oesophageal pathologies. Retrospective analysis of patient records identified 14 patients who had undergone subsequent endoscopy. Ten patients had endoscopic evidence of neo-Barrett's, columnar metaplasia was confirmed on histology in 8 cases but there were no cases of intestinal metaplasia. This study is limited in terms of size but it is important in that it represents the first description of neo-Barrett's in a paediatric surgical population. The authors state that symptoms were a poor indicator of pathology but the study is clearly underpowered to detect a significant difference.

The next study to report cases of neo-Barrett's in a paediatric population was published thirteen years later (Hamza et al., 2003). This retrospective case series from Egypt provides only very limited data on the cases of neo-Barrett's but it is by far the largest series of its type. Children underwent surgery for caustic strictures and reconstruction utilised a gastric 'pull-up' technique. The authors describe long term follow up of 75 patients with 10 cases of Barrett's identified and one case of carcinoma. The study is compromised by a lack of detail with regards to the follow up protocol and any statement as to whether there was routine endoscopic follow up. The duration of follow up is not stated and the authors do not state the definition of Barrett's oesophagus used or if

histological corroboration was obtained. With regards to the reported case of carcinoma it is unclear whether this was an adeno or squamous cell carcinoma or if there was any evidence of neo-Barrett's oesophagus in the patient concerned.

Borgnon and colleagues (2004) published a small case series of 21 children who had undergone oesophageal replacement with an isoperistaltic gastric tube. The surgery in this series differed from that undertaken in adults as in five cases of caustic stricture, the injured oesophagus was left in situ. All patients did however have an anastomosis between the stomach and the cervical oesophagus with exclusion of the oesophago-gastric junction. Nineteen patients underwent subsequent endoscopy and two cases of neo-Barrett's were identified. Unfortunately the authors of this study also fail to describe the diagnostic criteria used for neo-Barrett's and the timing and indications for endoscopic follow up are not stated.

The fourth study identified which considers neo-Barrett's in a paediatric surgical population was published by Spitz and colleagues (2004). This paper predominantly describes surgical techniques and outcomes but the authors recognise the possibility of neo-Barrett's following gastric 'pull-up' reconstruction. They state that they have encountered no cases in a series of 173 patients, the majority of whom had surgery for oesophageal atresia. The follow up protocol for these patients is not described and it is unclear how many of these children underwent endoscopy following surgery. This information is of critical importance given that Barrett's oesophagus and neo-Barrett's can only be diagnosed or excluded by endoscopic examination.

The most recent study available which considers Barrett's oesophagus after paediatric surgery was published in 2005 (Deurloo et al., 2005). This paper from the Netherlands involved children who had undergone surgery for oesophageal atresia. Unfortunately the surgical techniques are not described and it is therefore not possible to assess if the anatomy of these patients is similar to that of adults who have undergone oesophagectomy. Patients in this series were questioned about reflux symptoms and were invited to undergo endoscopic examination. Ninety two potentially eligible patients were identified

and 86 questionnaires were returned at a median follow up period of 17 years. Forty nine patients underwent endoscopy and 2 cases of columnar metaplasia were identified, both of gastric type.

In summary the available literature confirms that neo-Barrett's oesophagus can occur in children and young adults who have undergone oesophageal surgery and reconstruction with a gastric tube. The available literature is of insufficient quality to allow the prevalence or timing of this metaplasia to be determined.

2.7 Timescale for the development of neo-Barrett's

One of the unique features of the post-oesophagectomy human model for the development of Barrett's metaplasia is that it allows the timescale over which it develops to be determined. It is also possible to study the different subtypes of columnar metaplasia and the temporal relationship between them.

The earliest case of neo-Barrett's identified by the literature search occurred only 43 days after surgery (Franchimont et al., 2003). The authors describe a review of the resection specimen to exclude the presence of residual Barrett's and the histological examination to confirm the presence of columnar epithelium with specialised intestinal metaplasia. There are several other reported cases of neo-Barrett's occurring less than a year after surgery (O'Riordan et al., 2004)but no others at this very early stage. It is not clear whether there was clear correlation of endoscopic and histological findings in order to make the diagnosis raising the possibility of inadvertent sampling of the gastric conduit below the anastomosis rather than the tubular oesophagus above. The presence of intestinal metaplasia makes this less likely however, as one would not routinely expect to find this in a healthy gastric conduit.

Four studies were identified which sought to evaluate the association between time from surgery and the presence of neo-Barrett's (da Rocha et al., 2008, Nishimura et al., 2010, O'Riordan et al., 2004, Oberg et al., 2002). Nishimura and colleagues in their study of 100 patients describe no cases of columnar metaplasia at one month, 14% prevalence at one year and 40% prevalence in a subgroup of 58 patients who were followed up for two years. The authors state that this association was statistically significant quoting a p value of <0.05. The

other study which reports a significant association between the time from surgery and the presence of columnar metaplasia is that conducted by Da Rocha and colleagues (2008). As outlined above, patients in this study underwent regular endoscopic follow up and the prevalence of columnar metaplasia increased from 11% at 1-5 years (11/101) through to 30% between 5 and 10 years (18/61) and 58% for those more than 10 years post surgery. The authors state that this relationship is statistically significant but no p values are quoted.

Conversly, two studies report that there is no significant association between time from surgery and the presence of columnar metaplasia (O'Riordan et al., 2004, Oberg et al., 2002). Both of these studies were based on a single endoscopy for patients and comparison of time from surgery for those with and without columnar metaplasia. Interestingly in one study, (O'Riordan et al., 2004) median time from surgery for patients with columnar metaplasia was almost twice that of those with no metaplasia (39 months vs 20 months) yet this difference failed to reach statistical significance. The concern here must be that these studies, both of which involve less than 50 patients, may be underpowered to detect a difference in time from surgery.

Three studies were identified which address the relationship between time from surgery and the presence of neo-Barrett's with specialised intestinal metaplasia (Dresner et al., 2003, Oberg et al., 2002, da Rocha et al., 2008). All three of these found that there was a significant positive association. Dresner et al. (2003) compared the time to the development of non-specialised cardiac-type metaplasia (median 14 months) with the time to first detection of specialised intestinal metaplasia (median 27 months) and found a significant difference ($p=0.011$). Oberg et al. (2002) found that the median postoperative period was significantly longer in patients with intestinal metaplasia compared with those without (9.5 vs 4.2 years, $P=0.004$). Again there are concerns about the reliability of these findings as this study included only 3 patients with intestinal metaplasia. The third study to describe an association between time from surgery and the presence of specialised intestinal metaplasia is that by Da Rocha and colleagues (2008) but again, no statistical data is provided to support this claim.

Four studies describe progressive histological changes in individual patients (Lord et al., 2004, Gutschow et al., 2008, Dresner et al., 2003, D'journo et al., 2009). Lord describes one case where biopsies from the oesophageal remnant showed squamous epithelium at 15 months and cardiac type mucosa 9 months later. Unfortunately this study was based on retrospective evaluation of biopsy material and it is not clear how carefully the oesophageal remnant was examined for the presence of metaplasia at the first endoscopy. A similar case is described by Gutschow (2008) in which there was progression from squamous mucosa at 8 months to specialised intestinal metaplasia at 15 and 20 months and adenocarcinoma at 28 months. More convincing data of histological progression comes from the study by Dresner et al. (2003). In this prospective study, progression from squamous mucosa to cardiac type mucosa was demonstrated in 10 patients. In all cases, metaplasia was preceded by oesophagitis. The final study which reports histological progression is that by D'Journo and colleagues (2009). This is the only study to provide evidence that gastric type metaplasia might be a precursor to cardiac type metaplasia. The authors describe progression to cardiac type metaplasia in 4 of 12 patients who initially had gastric type metaplasia and progression to specialised intestinal metaplasia in 8 of 16 patients with cardiac type metaplasia.

In summary the data on the time to develop neo-Barrett's is inconsistent and tends to come from small studies which makes statistical analysis difficult. There are few reported cases occurring before one year. The available data tends to support the theory that the initial step is conversion to a non-intestinalised columnar epithelium with subsequent progression to specialised intestinal metaplasia.

2.8 Predisposing factors for the development of neo-Barrett's

2.8.1 Association with pre-operative histology

The most widely studied potential predisposing factor for neo-Barrett's is the pre-operative histology. Both the association with the tumour type and the presence of pre-operative Barrett's oesophagus have been assessed.

Only one study includes a multivariate analysis of potential predisposing factors (D'journo et al., 2009). This study considered the following potential predisposing factors; gender, age, previous Barrett's oesophagus, adenocarcinoma vs squamous cell carcinoma, neo-adjuvant therapy, thoracic anastomosis vs cervical anastomosis, anastomotic complications, proton pump inhibitor and prokinetic medication use. Previous Barrett's oesophagus was associated with a significantly increased risk of developing neo-Barrett's (odds ration 2.667). When this was considered using a multivariate model however, the threshold for statistical significance was not reached (p=0.064).

Other studies have assessed the association between the presence of pre-operative Barrett's oesophagus and the development of neo-Barrett's but all are based on univariate analysis. Oberg et al (2002) found that the prevalence of columnar metaplasia was significantly higher in patients with a pre-operative diagnosis of Barrett's oesophagus compared to others (69% vs 25%) but there were only 16 patients in each group. Two further studies have found no significant association between the presence of pre-operative Barrrett's oesophagus and the development of neo-Barrett's. One of these (Peitz et al., 2004b) involved only 14 patients and 10 cases of neo-Barrett's and it could be argued that statistical comparison of groups of this size is inappropriate. The second study by Franchimont and colleagues (2003) reported 7 cases of neo-Barrett's in 9 patients with a pre-operative diagnosis of Barrett's (77%) and 24 cases in 57 patients with no pre-operative diagnosis of Barrett's oesophagus (42%).(Franchimont et al., 2003) This difference was not statistically significant. It is important to note that this study included 35 patients with oesophageal adenocarcinoma so at least 26 patients in the group with no pre-operative Barrett's oesophagus actually had disease on the Barrett's metaplasia-dysplasia-adenocarcinoma spectrum.

Three studies were identified which evaluated the association between the original tumour type and the development of neo-Barrett's.(Dresner et al., 2003, Bax et al., 2007, O'Riordan et al., 2004) All of these employed univariate analysis only and all found that there was no statistically significant association between tumour type and neo-Barrett's.

2.8.2 Association with route of reconstruction

The type of surgical reconstruction and site of anastomosis following oesophagectomy has been identified as a potentially important factor. It has been suggested that a cervical anastomosis is associated with less reflux than an anastomosis in the chest (McKeown, 1976) and one might therefore expect the risk of neo-Barrett's to be lower in patients with a cervical anastomosis. Two studies were identified which assessed the route of reconstruction.

The study by D'Journo (2009) compared patients who had undergone an Ivor-Lewis procedure and thoracic anastomosis (n=36) with those who had undergone a 3-stage procedure with cervical anastomosis (n=48). The authors found that on multivariate analysis, a thoracic anastomosis was associated with a significantly greater risk of developing neo-Barrett's (Odds ratio 3.05, p=0.018). The second study to consider the route of reconstruction as a risk factor was that undertaken by Nishimura and colleagues (2010). This study compared the subcutaneous, retrosternal and posterior mediastinal routes of reconstruction but all patients are described as having a cervical oesophagogastrostomy and the surgical methods are not described in detail. No significant difference in the prevalence of neo-Barrett's related to the route of reconstruction was detected.

In summary the data on possible risk factors for neo-Barrett's is limited. Many studies are too small for good quality statistical analysis and only one study has employed multivariate analysis. There appears to be a trend towards an increased risk in patients with a pre-operative diagnosis of Barrett's oesophagus but statistical proof of this is lacking. Data from a single study suggests that a cervical anastomosis might be protective against neo-Barrett's.

2.9 Malignant progression in neo-Barrett's

The importance of Barrett's oesophagus lies in its association with oesophageal adenocarcinoma. Should neo-Barrett's have the same association it would mean that this finding might have clinical relevance for the patients involved. In addition this would provide further evidence that the post oesophagectomy model for the development of Barrett's is an accurate one.

Five papers were identified which described cases of dysplasia and adenocarcinoma arising within the oesophageal remnant following subtotal oesophagectomy. Two cases occurred more than 40 years after the initial surgical procedure. In both of these cases the original indication for surgery was a benign stricture in childhood. In one case progression was demonstrated from dysplastic neo-Barrett's through to invasive adenocarcinoma (Dunn et al., 2010). In the second case, intramucosal adenocarcinoma was present within the area of neo-Barrett's at the time of diagnosis (Lord et al., 2004).

Three further papers describe malignant progression in neo-Barrett's following surgery in adults. Da Rocha (2008) describes two cases of high grade dysplasia occurring in neo-Barrett's at 13 and 19 years following surgery. Both patients were followed up and went on to develop in situ adenocarcinoma over periods of 1 and 3 years respectively. This was managed with endoscopic mucosal resection. A detailed case report describes a case of neo-Barrett's occurring 15 months after subtotal oesophagectomy for a Barrett's adenocarcinoma (Gutschow et al., 2008). By 28 months this had progressed to invasive adenocarcinoma. The original proximal resection margin was free from metaplasia, effectively excluding the possibility of residual Barrett's or carcinoma.

Of most concern to clinicians caring for patients who have undergone oesophagectomy is the study by Wolfsen and colleagues (2004) which reports an exceptionally high incidence of dysplasia and adenocarcinoma in neo-Barrett's. In this study forty five patients were identified who had undergone oesophagectomy for Barrett's dysplasia or adenocarcinoma. Follow up periods for the cohort as a whole are not stated but the earliest patient underwent surgery 9 years prior to the publication of the paper. The study identified three cases of neo-Barrett's with low grade dysplasia, one case of neo-Barrett's with high grade dysplasia and two cases of neo-Barrett's in association with invasive adenocarcinoma. The proximal resection margin is described as being composed of squamous epithelium in all cases. Mean time to the diagnosis of LGD was 44 months, to HGD was 88 months and to adenocarcinoma was 13 months. There is clearly a marked discrepancy between the findings in this study and others which have reported outcomes following oesophagectomy.

No explanation for this is offered by the authors and there is no obvious reason why this cohort should differ from the others reported in the literature. Surgical methods employed were similar to those in other studies included in this review and proton pump inhibitors are described as being routinely prescribed. Patients were drawn from a North American population and the only marked difference between this and other series appears to be the inclusion only patients who underwent surgery for adenocarcinoma or dysplastic Barrett's oesophagus.

One case report of adenocarcinoma occurring after surgery for tracheo-oesophageal fistula (TOF) was identified (Alfaro et al., 2005). The case described is of a 46 year old female who developed adenocarcinoma on a background of extensive Barrett's oesophagus at the age of 46 following surgery as an infant. The type of surgery involved is not described and it is therefore not possible to determine if this represents a situation analogous to that occurring after subtotal oesophagectomy. Gastric conduits may be used in the repair of TOF, particularly where this is associated with long-gap oesophageal atresia but in other cases primary closure is the norm (Arul GS, 2008).

In summary 7 cases of adenocarcinoma arising within neo-Barrett's were identified by this literature search with a further 4 cases of dysplasia. One case of adenocarcinoma following surgery for tracheo-oesophageal fistula was also identified. There is low grade evidence from case reports to show malignant progression which indicates that the metaplasia-dysplasia-adenocarcinoma sequence can occur in the context of neo-Barrett's. Given the small numbers of patients involved it is not possible to accurately determine the risk of malignant progression in neo-Barrett's.

2.10 Molecular marker expression in neo-Barrett's

This literature search identified four studies which involved the assessment of molecular marker expression in neo-Barrett's tissue. A variety of markers were evaluated and the number of samples assessed tended to be small.

The largest study of this type was published in 2007 (Bax et al., 2007). Eighteen patients with neo-Barrett's were identified including 7 with specialised intestinal metaplasia (SIM). Immunohistochemisty was used to assess for the presence of CDX2, MUC2 and cytokeratins 7 and 20 and CDX2 expression was further evaluated by polymerase chain reaction techniques. CDX2 is a protein involved in intestinal differentiation, predominantly expressed in the small intestine and colon of adults which is also known to be present in Barrett's tissue. CDX2 expression was found in all 7 patients with specialised intestinal metaplasia and in a further 2 samples from patients with gastric type metaplasia. The authors suggest that this indicates that gastric metaplasia is related to specialised intestinal metaplasia. MUC2 is a mucin protein normally found in the intestine which has also been found in Barrett's oesophagus. In the neo-Barrett's tissue studied MUC2 was observed in the goblet cell cytoplasm of all patients with SIM, it was not found in any patients with gastric type metaplasia. Cytokeratins 7 and 20 were present in all samples of neo-Barrett's regardless of subtype but the exact staining pattern is not described and it is therefore difficult to compare this to the pattern which has been reported in sporadic Barrett's oesophagus.

The second largest study to assess molecular marker expression in neo-Barrett's involved samples from only 10 patients (Lord et al., 2004). This study included a more detailed examination of cytokeratin 7 and 20 staining pattern using immunohistochemical techniques. Cytokeratins are the intermediate filaments characteristic of epithelial cells and they occur in several different forms. Sporadic Barrett's epithelium is characterised by strong CK7 staining at the surface and in deep glands and weak superficial CK20 staining (Ormsby et al., 1999). This study by Lord and colleagues found that CK7 and CK20 staining patterns were similar in the neo-Barrett's samples to what is reported in sporadic Barrett's. This held true regardless of the subtype of neo-Barrett's. This study also used immunohistochemistry to evaluate the expression of DAS1, topoisomerase 2α, cycloxygenase 2 (COX-2) and ornithine decarboxylase (ODC). The authors chose DAS1 as a further comparison against sporadic Barrett's as this antibody is known to react against Barrett's cells but not against normal oesophageal mucosa. DAS1 stained the mucin in the goblet cells of neo-Barrett's with SIM intensely and also faintly stained the

cytoplasm of some columnar cells in neo-Barrett's without SIM. Topoisomerase 2α was used to assess cellular proliferation and COX-2 and ODC to assess the potential for dysplasia. Overall the authors of this study conclude that the expression profile of neo-Barrett's is similar to that found in Barrett's oesophagus and different from that of normal oesophageal or gastric mucosa. The main weakness of this study is the small numbers of samples involved.

Chaves and colleagues (2002) used an antibody against the enterocytic enzyme sucrase-isomaltase (SI) to assess for intestinal type differentiation in samples from 4 patients with neo-Barrett's. There was no direct comparison with Barrett's tissue, but the authors do make reference to a previous paper from the same unit. In that study this enzyme was found to be present in 8 of 12 cases of Barrett's oesophagus studied. In the 4 neo-Barrett's patients only one case of unequivocal SI staining was identified but really the numbers involved in both this study and the original study of sporadic Barrett's are too small for this to be a meaningful comparison.

The final paper identified which assessed molecular marker expression in neo-Barrett's was the case report by Gutschow and colleagues (2008). Here the authors studied expression of three markers in samples from a single patient who progressed from squamous epithelium through to neo-Barrett's metaplasia and invasive adenocarcinoma. The markers were COX-2, BCL-2, a protein involved in regulating apoptosis and survivin, another protein involved in regulating apoptosis. Quantitative real-time polymerase chain reaction was used to measure expression of these genes and a stepwise increase in each was found as progression to adenocarcinoma occurred.

Overall there is very limited evidence available on molecular marker expression in neo-Barrett's. The evidence that has been published is from small studies and the markers investigated are varied. There is insufficient data to conclude whether neo-Barrett's is characterised by the same molecular markers as sporadic Barrett's oesophagus.

2.11 Summary

The major finding of this review is that there are very few good quality studies looking at neo-Barrett's metaplasia occurring in the oesophageal remnant following oesophagectomy. Most published series involve small numbers of patients and this has made the evaluation of potential risk factors for neo-Barrett's difficult. There is insufficient data to assess the risk of malignant progression in neo-Barrett's and this is critical if we are to be able to devise evidence based follow-up protocols for patients. Data on molecular marker expression in neo-Barrett's is extremely limited. In order to confirm the accuracy of the post-oesophagectomy human model for the development of Barrett's significantly more work in this area is required. These issues provide the indication for the present study.

2.12 Aims of the study

The aims of this study are:

1. To establish the incidence of post-oesophagectomy neo-Barrett's in a large series of patients and the timescale over which this develops

2. To establish whether or not neo-Barrett's oesophagus is characterised by expression of the same cellular proteins as sporadic Barrett's oesophagus

3. To establish whether or not genetic mutations present in the original tumour or Barrett's oesophagus are present in post-operative neo-Barrett's oesophagus

2.13 Hypotheses

Neo-Barrett's metaplasia is a de novo phenomenon and is unrelated to the disease process in the oesophagus pre-operatively.

Neo-Barrett's metaplasia in the remnant oesophagus is an inevitable consequence of subtotal oesophagectomy and reconstruction with a gastric conduit.

Neo-Barrett's metaplasia occurring after oesophagectomy is characterised by the same cellular proteins as sporadic Barrett's and is an accurate model for this.

Neo-Barrett's metaplasia has the potential to progress to dysplasia

Chapter 3. A study of the incidence of post-oesophagectomy Barrett's and the timescale over which it develops

3.1 Introduction

In Barrett's oesophagus the squamous epithelium of the distal oesophagus is replaced with metaplastic columnar epithelium. The condition is acquired in response to the harmful effects of acid and bile refluxing into this region (Vaezi and Richter, 1996). Whilst reflux is now well recognised as the major aetiological factor in Barrett's oesophagus the precise mechanisms underlying the pathogenesis remain incompletely understood.

Following subtotal oesophagectomy with a gastric conduit reconstruction the majority of patients experience reflux of gastric and duodenal contents (Aly and Jamieson, 2004). *In vivo* ambulatory studies in Newcastle (Dresner et al., 2003) have demonstrated abnormal oesophageal exposure to both acid and bile over a 24 hour period in over 80% of post operative patients. Elsewhere, Öberg and colleagues (2002) have reported abnormal oesophageal acid exposure in 78% of patients. The high prevalence of reflux results from the disruption of the normal anatomical anti-reflux mechanisms (figure 3.1). The lower oesophageal sphincter, angle of His and diaphragmatic sling are all resected or disrupted. Many surgeons perform a routine pyloroplasty to facilitate gastric emptying but this has the associated adverse effect of promoting duodenal reflux. The position of the gastric tube between the positive pressure environment of the abdominal cavity and the negative pressure of the thoracic cavity further promotes reflux.

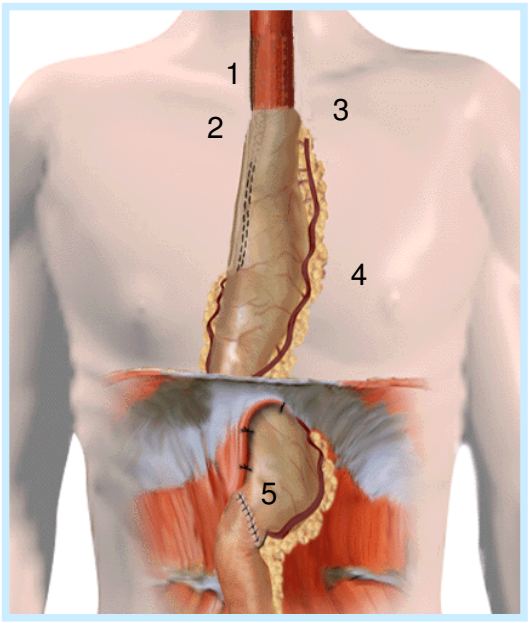
Since 1977 it has been recognised that columnar metaplasia can develop above an oesophago-gastric anastomosis and this has been termed neo-Barrett's (Hamilton and Yardley, 1977). This phenomenon is potentially clinically important for the patients affected. It also provides a unique opportunity to study the development of Barrett's oesophagus in a situation which avoids some of the pitfalls associated with observations in sporadic Barrett's oesophagus.

It is not normally possible to ascertain the timescale over which Barrett's develops in response to reflux. In the post-oesophagectomy patient, the operation date represents a baseline and allows a timescale to be determined for the development of neo-Barrett's with examination of the resection specimen

confirming squamous epithelium at the site of anastomosis which is unlikely to have been exposed to significant reflux pre-operatively.

Segments of neo-Barrett's have the same endoscopic appearance as standard Barrett's and the surgical anastomosis is easily identified at endoscopy. Any biopsies from above this anastomosis are clearly oesophageal in origin and the difficulties normally associated with the accurate identification of the gastro-oesophageal junction are eliminated in this group of patients.

The aim of this study was to prospectively evaluate the incidence of neo-Barrett's in a large cohort of patients, to define the timescale over which it develops and to assess possible predisposing factors.



1. Absence of lower oesophageal sphincter

2. Absence of normal acute angle of His

3. Absence of diaphragmatic 'pinch' effect at gastro-oesophageal junction

4. Gastric conduit positioned between positive pressure abdominal cavity and negative pressure thoracic cavity

5. Pyloroplasty

Figure 3.1: Anatomical features leading to reflux after subtotal oesophagectomy (reproduced from Minimally Invasive Esophagectomy, (Luketich et al., 2000), with kind permission of Elsevier)

3.2 Patients and Methods

3.2.1 Study population

Patients for this study were enrolled from the Northern Oesophago-gastric cancer unit. This unit, based in Newcastle upon Tyne, provides treatment for

patients with oesophageal carcinoma from across the north of England. It is one of the largest such units in Europe undertaking between 60 and 75 subtotal oesophagectomies per year.

Endoscopic evaluation of the oesophagogastric anastomosis and the remnant oesophagus is now a routine aspect of post-operative follow up in the unit. The follow up protocol is designed to identify patients who develop neo-Barrett's and to allow the option of surveillance to be considered. The current protocol is to endoscope patients at around 1 year following surgery and at 5 years following surgery. Early endoscopy aims to identify those with rapid development of neo-Barrett's who may be at higher risk of progression. Delayed endoscopy aims to identify patients who are considered 'cured' but who have neo-Barrett's and may benefit from long term follow up and surveillance.

Historically follow-up endoscopy was not routine and many patients therefore undergo examination outside of the above protocol. In addition patients with new onset upper gastro-intestinal tract symptoms undergo urgent endoscopic evaluation. Very early endoscopy is occasionally undertaken when an anastomotic stricture is suspected. Patients with known or suspected distant disease recurrence do not routinely undergo endoscopy. Patients in whom endoscopy is not felt to be clinically relevant by the named consultant are also excluded from the above protocol. Examples of this would be patients who are particularly frail.

3.2.2 Surgical technique

All surgery is performed by, or under the direct supervision of, a consultant with a specialist practice in oesophago-gastric surgery and the detailed resection techniques have been described elsewhere (Griffin SM and Raimes SA, 2006). The main steps of the operation include an upper midline laparotomy through which the stomach is mobilised based on a vascular pedicle of the right gastric and gastroepiploic arteries. The lesser omentum is divided encompassing nodes along the lesser curve and those at the origin of the coeliac trunk. The common hepatic artery and the roots of the splenic and left gastric arteries are also skeletonised by complete removal of the surrounding nodal tissue. The left gastric artery is divided at its origin and an en bloc hiatal dissection is performed

removing the left and right paracardial nodal stations together with the respective crura. A pyloroplasty is routinely performed to ensure adequate gastric drainage.

Via a fifth intercostal space right thoracotomy the oesophagus is mobilised with any connective tissue and the encompassing mediastinal pleura dissected off the aorta and pulmonary veins. A meticulous lymphadenectomy of the paratracheal, carinal and left and right bronchial nodes is performed followed by *en bloc* excision of the thoracic duct and para-aortic nodes. The oesophagus is transected at the level of the thoracic inlet and the stomach delivered into the chest. Sleeve resection of the lesser curve and the associated nodes is undertaken and a stapled oeosophago-gastric anastomosis fashioned. The nodes in the aorto-pulmonary window are removed but neither a full dissection of the left recurrent laryngeal nerve chain nor a cervical lympadenectomy are routinely undertaken.

3.2.3 Historical data

Data from the pre-operative staging process was obtained from a prospectively compiled database held within the Northern Oesophago-Gastric Cancer Unit.

3.2.4 Ethical approval

Ethical approval for the study and for additional biopsies to be taken during routine endoscopy was sought and granted by the County Durham and Tees Valley 2 REC (reference number 08/H0908/25, Appendix 1). Reseach and Development approval was also granted by the Newcastle upon Tyne Hospitals NHS Foundation Trust.

3.2.5 Patient assessment

Patients attending for endoscopy were questioned about the long-term use of proton-pump inhibitors, pro-kinetics, aspirin and non-steroidal anti-inflammatory (NSAID) medications since the time of surgery. The presence or absence of reflux symptoms was noted although no attempt was made to quantify these symptoms. Medical notes were reviewed in order to obtain the results of any previous post-operative endoscopies.

3.2.6 Consent and study information

Routine informed consent for endoscopy was sought prior to examination. After ethical approval to take additional biopsies for research purposes had been granted patients were issued with an information sheet regarding the study prior to their endoscopy (Appendix 2). Patients were asked whether they wished to participate in this study and additional written consent for this was obtained.

3.2.7 Endoscopic assessment

All patients underwent endoscopic assessment by, or in the presence of the same endoscopist. A range of Olympus video-endoscopes were used with external diameters ranging from 9.0 – 11millimetres (XQ260, XQ240, H260, 1T240, Olympus KeyMed, UK). Either topical pharyngeal anaesthesia with 10% lidocaine (Xylocaine®, AstraZeneca, Luton, UK) or intravenous sedation with 2 - 5 milligrams of midazolam (Hameln, Gloucester, UK) were used according to patient preference. Patients were allowed to continue all usual medications up to and including the day of the test.

With the patient in the left lateral position the endoscope was introduced and the distance from the incisors to the anastomotic line was measured. The presence or absence of endoscopic columnar epithelium above the anastomosis was noted before the gastric conduit, pylorus and duodenum were examined. The anastomosis was also viewed on retroflexion and the distance from the incisors was confirmed as the endoscope was withdrawn.

3.2.8 Biopsy protocol

Standard biopsy protocol for post oesophagectomy patients with endoscopic neo-Barrett's is as follows:-

2 or more biopsies from the area of columnar epithelium within the tubular oesophagus above the surgical anastomosis
2 biopsies from the neo-cardia 2cm below the anatstomotic site

Where there is no endoscopic evidence of neo-Barrett's biopsies are undertaken at the discretion of the endoscopist. Patients who are anti-coagulated do not undergo routine biopsy.
Patients consenting to additional research biopsies had an additional 4 biopsies taken from the area of endoscopically visible columnar epithelium above the surgical anastomosis. They also had an additional 4 biopsies taken from the neo-cardia and 2 biopsies taken from any area of endoscopically normal squamous epithelium above the squamo-columnar junction.

All biopsies were taken using standard spiked biopsy forceps (Radial Jaw 3, Boston Scientific, MA, USA).

The diagnosis of Barrett's oesophagus relies upon histological corroboration of endoscopic findings. The presence of the visible surgical anastomosis allows the endoscopist to be confident that biopsies taken from above this are oesophageal in origin. No specific height above the anastomosis was set for biopsies as this would prevent the evaluation of short segments of columnar metaplasia.

3.2.9 Processing of biopsy material

All biopsy specimens were immediately placed onto strips of filter paper. Specimens to be embedded in paraffin were transported in 10% neutral buffered formalin. Specimens to be snap-frozen were transported wrapped in damp gauze in universal containers. The strips of tissue were processed for embedding in paraffin. This was done in an automated tissue-processing machine according to the protocol described in Appendix 3. Serial sections of 5 micrometres were cut using the microtome and sections were de-waxed and rehydrated according to the protocol described in Appendix 4.

3.2.10 Histopathological assessment and definitions

Biopsies for histological assessment were stained with Haematoxylin and Eosin using a standard automated system. Biopsy results were reported by the pathology department at the Royal Victoria Infirmary, Newcastle. All supra-anastomotic biopsies demonstrating columnar epithelium were reviewed by an experienced gastro-intestinal pathologist.

For the purposes of this study the definition of neo-Barrett's epithelium included all types of columnar epithelium in biopsies taken from the remnant oesophagus. Columnar epithelium was subdivided into 3 types as originally described by Paull et al. (1976).

A Columnar epithelium with specialised intestinal metaplasia

B Specialised gastric mucosa with parietal cells (body or fundic type)

C Non-specialised gastric mucosa (cardiac type)

3.2.11 Statistical analysis

Categorical data were analysed using the chi-squared or Fisher's exact test as appropriate. Numerical data were analysed using either the independent students t-test or the Mann-Whitney U test.

3.3 Results

3.3.1 Study population

134 patients who had previously undergone subtotal oesophagectomy and reconstruction with as gastric conduit underwent prospective endoscopic evaluation during the course of the study.

3.3.2 Exclusions

Patients were excluded from the study if there was evidence of residual Barrett's oesophagus at the proximal resection margin of the surgical specimen (7 patients). Patients were also excluded if there was evidence of local tumour recurrence (1 patient).

3.3.3 Demographics

A total of 126 patients were therefore included in the study population. The male to female ratio was 2.15:1. Median age at the time of endoscopy was 67 (range 20 – 85).

3.3.4 Indications for Original Resection

The indications for original resection are given in table 3.1. The one case of benign histology relates to a patient with squamous cell carcinoma on pre-operative biopsies. No evidence of squamous cell carcinoma was found in the resection specimen but subsequent re-evaluation of the biopsies by a second pathologist confirmed the original pre-operative diagnosis. One patient had high grade dysplasia in a small nodule at the gastro-oesophageal junction, there was no endoscopic or histological evidence of Barrett's metaplasia above the gastro-oesophageal junction.

In all cases where there was evidence of neo-Barrett's the original resection specimen was retrieved and reviewed by an experienced gastro-intestinal pathologist to exclude the presence of residual Barrett's metaplasia at the proximal resection margin.

Resection Specimen Histology	Number of patients	Clear endoscopic evidence of Neo-Barrett's	Confirmed Neo-Barrett's
Adenocarcinoma	83	34	33
Squamous Cell Carcinoma	22	5	5
High Grade Dysplasia in Barrett's	10	4	4
High Grade Dysplasia in Squamous epithelium	2	0	0
Gastro-intestinal Stromal Tumour (GIST)	5	1	1
Undifferentiated carcinoma	1	0	0
Hamartoma	1	1	1
Benign	1	1	1
High Grade Dysplasia in nodule at gastro-oesophageal junction	1	0	0
Total	126	46	45

Table 3.1: Indications for surgery in study patients (confirmed Neo-Barrett's defined as endoscopic evidence of columnar metaplasia with histological corroboration)

3.3.5 Prevalence of pre-operative Barrett's Oesophagus

Barrett's oesophagus was noted at the pre-operative staging endoscopy in 54 patients. In ten of these patients high grade dysplasia within Barrett's oesophagus was the primary indication for surgery. A further 43 patients had endoscopic evidence of Barrett's oesophagus along with adenocarcinoma. This equated to 52% of the 83 patients who underwent surgery primarily for adenocarcinoma. Only one patient with a non-Barrett's/adenocarcinoma indication for surgery was noted to have endoscopic evidence of Barrett's oesophagus pre-operatively.

Fifty six patients had histological evidence of Barrett's oesophagus in the resected specimen. All patients with histological evidence of Barrett's oesophagus had undergone resection for either high grade dysplasia in Barrett's or adenocarcinoma. Forty six of eighty three patients (55%) with adenocarcinoma had histological evidence of Barrett's oesophagus along with

the tumour in the resected specimen. No patient with a non-Barrett's/adenocarcinoma indication for surgery was noted to have histological evidence of Barrett's oesophagus.

3.3.6 Timing of endoscopy

The median time from surgery to endoscopic evaluation was 3.6 years (range 0.3 – 13.2 years. Twenty patients went on to have a second endoscopy during the study period, one patient had three endoscopic examinations.

3.3.7 Endoscopic findings

Forty six patients (37%) had clear endoscopic evidence of neo-Barrett's oesophagus above the surgical anastomosis. An additional four patients had possible neo-Barrett's but either food residue or marked inflammation made the appearances difficult to interpret. One patient had recurrent adenocarcinoma at the anastomosis 15 months after surgery.

Several patterns of neo-Barrett's were recognised:

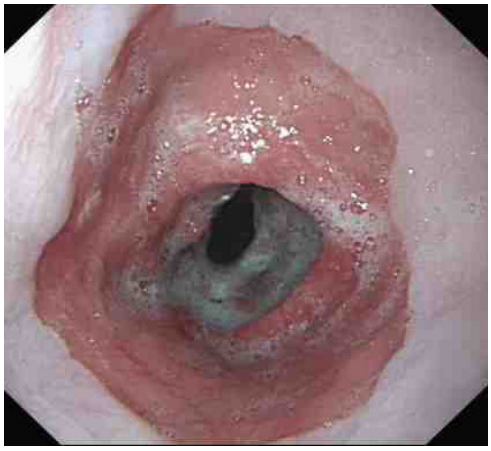
1. Circumferential segment
2. Single tongue or tongues measuring at least 1cm
3. Small encroachments of columnar epithelium less than 1cm in length

The incidence of each pattern of neo-Barrett's are shown in table 3.2. Examples of these patterns are shown in figure 3.2

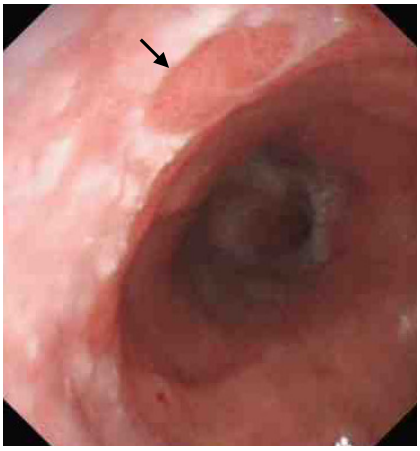
Pattern of neo-Barrett's	Number of cases
Circumferential segment	19
Tongues	17
Small encroachment	8
Not recorded	2

Table 3.2: Incidence of each endoscopic pattern of neo-Barrett's

a



b



c

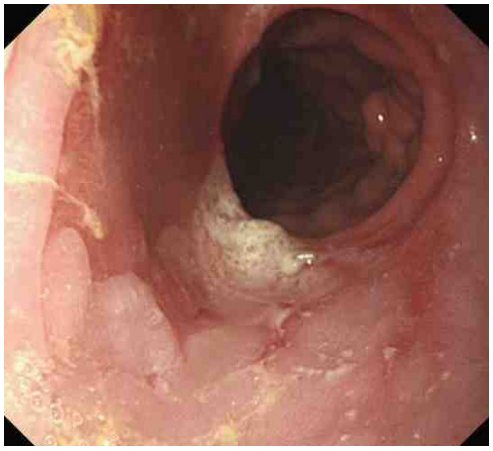


Figure 3.2: Endoscopic patterns of neo-Barrett's; a – Circumferential segment, b – Small encroachment of columnar epithelium, c – Tongues of columnar epithelium

3.3.8 Histological findings

Ninety seven patients (77%) had supra-anastomotic biopsies taken. The histological findings are summarised in table 2.2. Twenty nine patients had no supra-anastomotic biopsies taken. The majority of these patients (27/29) had a healthy oesophageal remnant, biopsies were not felt to be clinically indicated and would therefore not have been covered by the ethical approval for this study. The remaining two patients had endoscopic evidence of neo-Barrett's but biopsy confirmation was not possible. In one case this was due to anti-coagulation and in the other case the procedure was poorly tolerated and biopsy was felt to be unsafe and inappropriate.

Histological Findings	Number of patients
Columnar metaplasia	46
Squamous epithelium	49
Recurrent adenocarcinoma	1
Ulcer material only	1

Table 3.2: Histological findings in supra-anastomotic biopsies

3.3.9 Incidence and classification of Neo-Barrett's

The British Society of Gastroenterology guidelines (2005) define Barrett's oesophagus as a segment of columnar metaplasia of any length visible endoscopically above the oesophago-gastric junction and confirmed or corroborated histologically. For the purposes of this study neo-Barrett's is defined as a segment of columnar metaplasia visible endoscopically above the surgical oesophago-gastric anastomosis which is confirmed or corroborated histologically. The overall incidence of Neo-Barrett's in this study at a median follow up of 3.6 years was 35.7% (45/126).

Many patients had more than one type of epithelium present in biopsy samples including fragments of squamous epithelium. Patients with any evidence of intestinal metaplasia were classified as type A. Otherwise patients were classified according to the predominant subtype. All three described subtypes of columnar metaplasia were observed in this study. Table 2.3 details the incidence of each subtype. The incidence of Neo-Barrett's with specialised intestinal metaplasia was 10%, an example of this epithelium is shown in figure 3.3.

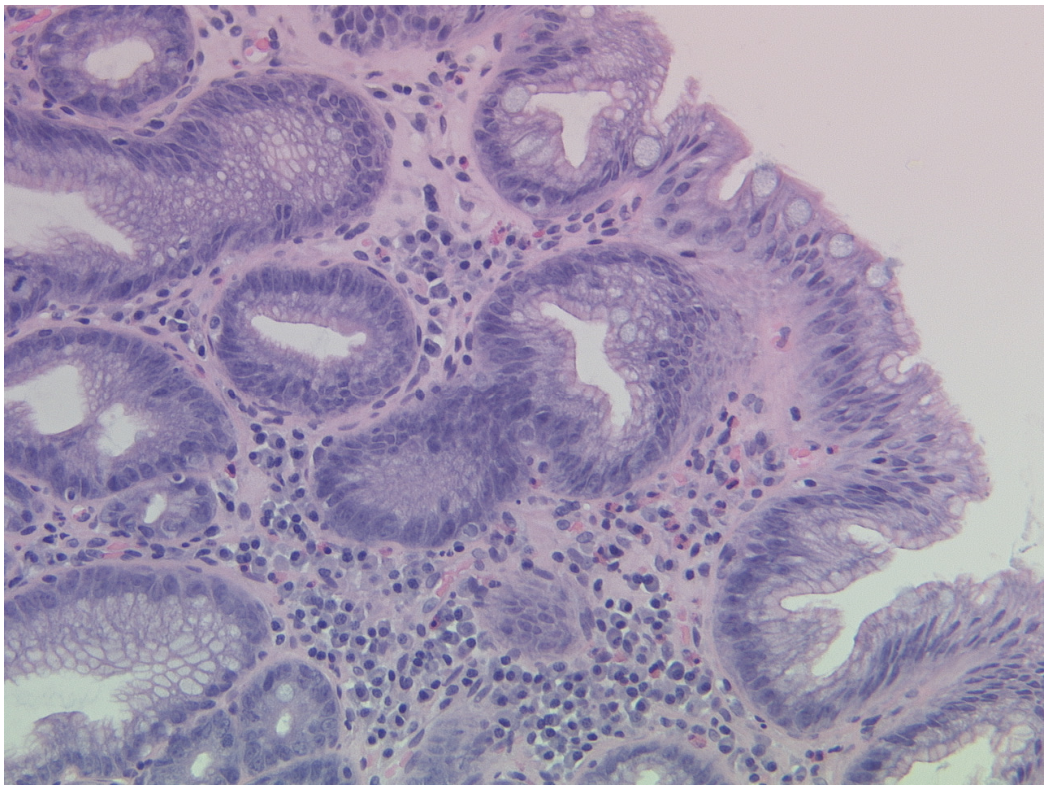


Figure 3.3 Histological image of neo-Barrett's with specialised intestinal metaplasia (Type A)

Type of columnar metaplasia	N
A - specialised intestinal metaplasia	12
B – body or fundic type metaplasia	17
C – cardiac type metaplasia	16

Table 3.3: The incidence of the subtypes of columnar metaplasia in patients with neo-Barrett's

3.3.10 Length of Neo-Barrett's segment

At the first study endoscopy 45 patients had confirmed neo-Barrett's. Both circumferential (n=20) and non-circumferential (n=25) types of Barrett's were observed. Median maximal length of the Barrett's segment was 1.5cm (range <1cm-8cm). Where a circumferential segment was present the median length of this was 2.75cm (range <1 – 8cm).

During the study period 15 patients with confirmed neo-Barrett's at first endoscopy underwent a second endoscopy at a median of 12 months following the first endoscopy (range 9-19 months). In 13 cases (87%) neo-Barrett's was also noted at the second endoscopy, in one case (initially <1cm in length) there appeared to have been regression of the columnar metaplasia. In the remaining one case, food debris meant that accurate assessment was impossible. Where a neo-Barrett's segment was measured on more than one occasion there was little individual variation in maximum length and the median was unchanged at 3cm.

3.3.11 Time following oesophagectomy

The median post-operative period was significantly longer in patients with confirmed neo-Barrett's compared to those with no evidence of neo-Barrett's (5.72 vs. 2.21 yrs, p=<0.001). The earliest confirmed case of neo-Barrett's was noted at 9 months following oesophagectomy. Figure 2.4 shows the prevalence of columnar metaplasia in patients with varying lengths of time between surgery and endoscopy. The prevalence is seen to increase with time.

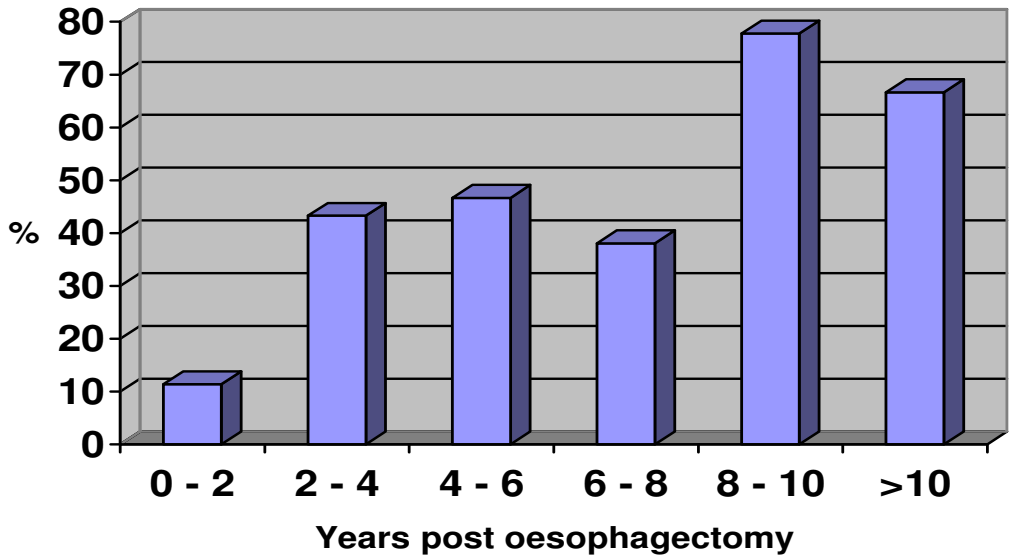


Figure 3.4: Prevalence of columnar metaplasia within the oesophageal remnant in patients with varying lengths of time between surgery and endoscopy

3.3.12 Association between histological subtype and time

Mucosal biopsies were classified into one of three histological subtypes as detailed above. Median time from surgery to the finding of each subtype was 8.1 years (Type A, specialised intestinal metaplasia), 5.1 years (Type B, body or fundic type), 4.4 years (Type C, cardiac type) (figure 3.5). The time elapsed between surgery and index study endoscopy was significantly greater for patients with specialised intestinal metaplasia (SIM, Type A) compared to those with columnar metaplasia without SIM (Types B and C), (8.1 years vs 4.8 years, p=0.025) (figure 3.6). There was no significant difference in the time elapsed from surgery to the finding of either of the two types of non-intestinalised metaplasia, p=0.449.

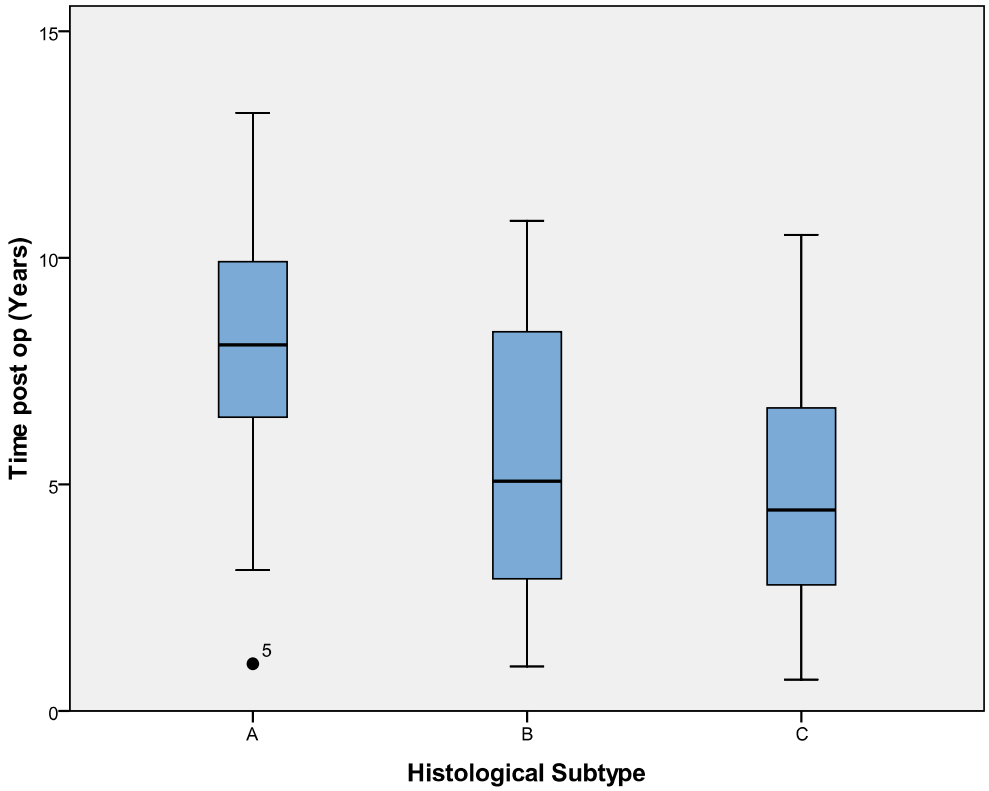


Figure 3.5: Box plot of the time elapsed following surgery and the histological subtype of neo-Barrett's

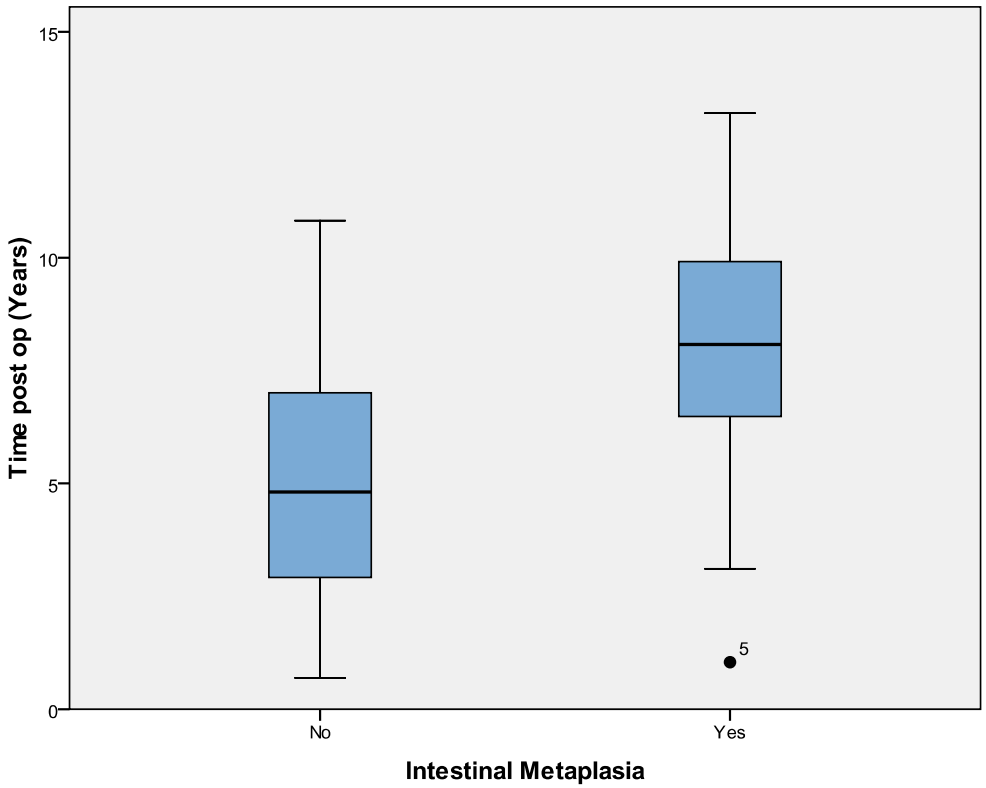


Figure 3.6: Box plot of the time elapsed following surgery and the presence of neo-Barrett's with and without specialised intestinal metaplasia.

3.3.13 Progression between histological subtypes

Twenty one patients underwent a second endoscopy during the study period. No progression from squamous epithelium to columnar metaplasia was observed during the study period in the 7 patients with no neo-Barrett's. In patients with confirmed neo-Barrett's at index endoscopy there was no evidence of progression from columnar metaplasia without SIM to columnar metaplasia with SIM. Two patients with SIM on initial biopsies had no evidence of SIM in repeat biopsies.

3.3.14 Association with clinical and pathological features

Several potential pre-disposing factors for neo-Barrett's were assessed. In all cases, patients with confirmed neo-Barrett's (i.e. endoscopic evidence with histological corroboration) were compared with those with no evidence of neo-Barrett's. Cases where there were conflicting endoscopic and histological findings or where biopsy to confirm the diagnosis was not possible were excluded (n=6).

3.3.15 Association with original histological subtype

The incidence of neo-Barrett's following resection for adenocarcinoma or high grade dysplasia in Barrett's oesophagus was 41% (37/90). The incidence of neo-Barrett's following resection for disease not on the Barrett's metaplasia-dysplasia-adenocarcinoma spectrum was 27% (8/30) (Figure 3.7). This difference was not statistically significant (Chi square) (p=0.157).

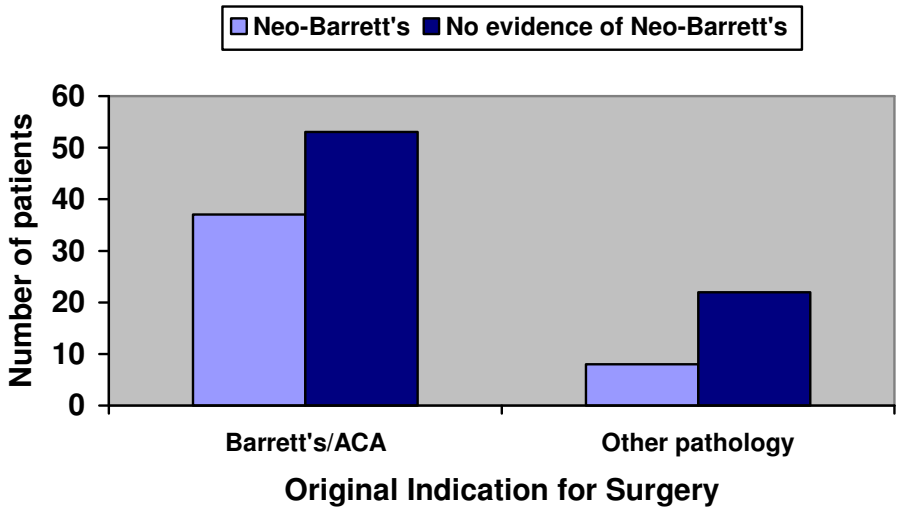


Figure 3.7: Prevalence of neo-Barrett's according to the original indication for surgery

3.3.16 Association with previous Barrett's Oesophagus

The incidence of neo-Barrett's in patients who had evidence of Barrett's oesophagus described in the original resection histology report was 45% (25/55). The incidence of neo-Barrett's in patients with no previous evidence of Barrett's oesophagus was 31% (20/65), again this difference was not statistically significant (p=0.098)(figure 3.8).

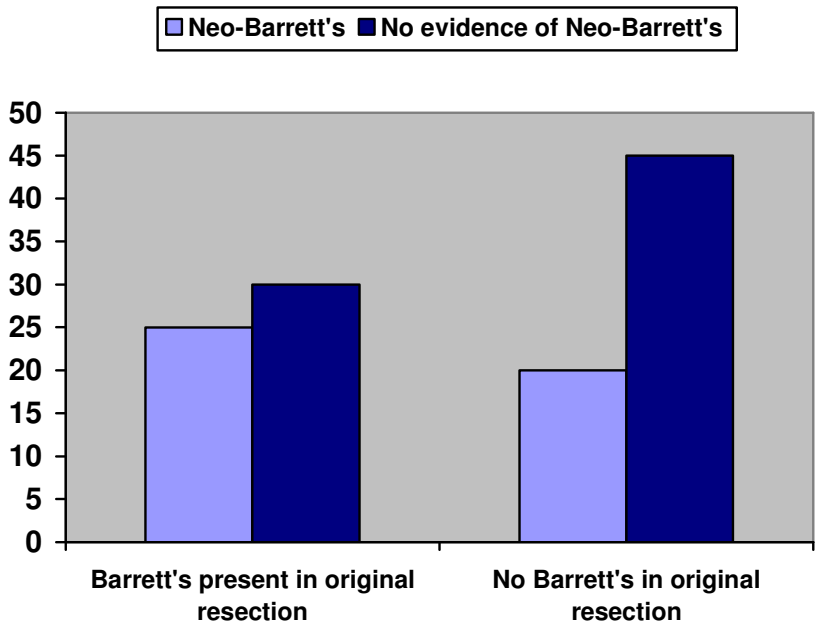


Figure 3.8: Prevalence of neo-Barrett's according to the presence of Barrett's oesophagus in the original resection specimen

3.3.17 Patient gender and Neo-Barrett's

The incidence of neo-Barrett's in male patients was 40% (34/84). The incidence of neo-Barrett's in female patients was 31% (11/36). This did not represent a significant difference (p=0.304).

3.3.18 Symptomatic reflux, proton pump inhibitor use and Neo-Barrett's

Seventy nine patients (66%) reported either intermittent or ongoing reflux symptoms in the post operative period or had reflux symptoms recorded in their notes at the time of previous clinic visits. There was no significant association between the presence of reflux symptoms and the presence of neo-Barrett's (p=0.518). The majority of patients were either taking PPI at the time of their endoscopy or reported having taken a PPI for the majority of time following their surgery. The incidence of neo-Barrett's was significantly higher in patients with no history of long term PPI use 67% (12/18) vs 32% (33/103) (p=0.006).

3.3.19 Height of surgical anastomosis

The distance from the incisors to the surgical anastomosis was recorded for 106 patients. The median distance was identical for those with and without neo-Barrett's at 24cm (range 18-34cm) (figure 3.9). Where more than one

endoscopy was conducted the level recorded at the first study endoscopy was used in analysis.

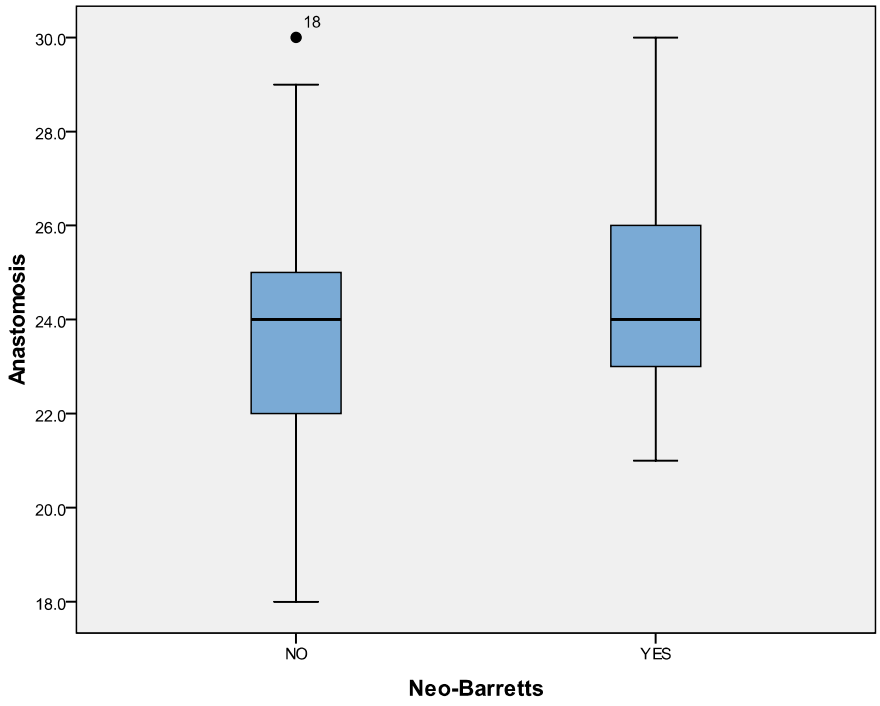


Figure 3.9: Box plot showing the distance at endoscopy from the incisors to the surgical anastomosis in centimetres in patients with and without neo-Barrett's

3.4 Discussion

Metaplastic columnar epithelium occurring in the oesophageal remnant following oesophagectomy and reconstruction with a gastric conduit is a well recognised phenomenon. In the present study the overall incidence of neo-Barrett's was 36% (45/126) at a median follow up of 3.6 years. This finding provides corroborative evidence for the high incidence of neo-Barrett's reported in previous smaller studies.

Sporadic Barrett's oesophagus is generally described as a relatively rare condition but the true population prevalence is difficult to determine as the diagnosis can only be made endoscopically. A figure of 1-2 % is most commonly given (Ronkainen et al., 2005, Watson et al., 2005) but figures vary widely and inconsistent diagnostic definitions have been used. One study of asymptomatic American males attending for colorectal cancer screening found the incidence of Barrett's oesophagus to be 25% (Gerson et al., 2002).

Neo-Barrett's oesophagus appears to be far more common in post-oesophagectomy patients than sporadic Barrett's oesophagus is in the general population. Since Hamilton and Yardley's first description (1977), several studies have all have found the incidence to be much higher than the generally accepted rate of Barrett's oesophagus with incidence rates of 22-93% described (Bax et al., 2007, da Rocha et al., 2008, D'journo et al., 2009, Dresner et al., 2003, Peitz et al., 2004b, Wolfsen et al., 2004, Lord et al., 2004, O'Riordan et al., 2004, Oberg et al., 2002). This group of studies is very heterogeneous. The indications for surgery, follow up periods and precise definitions of neo-Barrett's used all vary. These factors are likely to contribute to the wide range of incidences reported.

Few surgical centres routinely endoscope patients during follow up making comparisons between data series difficult. Retrospective review of endoscopy records is the basis of several studies..(Bax et al., 2007, Franchimont et al., 2003, Wolfsen et al., 2004, Lord et al., 2004) In these cases, endoscopy would seem more likely to have been undertaken in symptomatic individuals, potentially those with severe reflux, giving rise to a source of potential selection bias.

Prospective assessment of patients in a research setting, specifically looking for neo-Barrett's has been undertaken by several groups.(D'journo et al., 2009, Dresner et al., 2003, O'Riordan et al., 2004, Oberg et al., 2002) These studies report some of the highest incidence rates of neo-Barrett's. Increased awareness and recognition of neo-Barrett's amongst the endoscopists involved in these studies may explain the higer incidence observed. It is also possible that there is a degree of bias in these studies due to patient self selection, with those most troubled by reflux being most likely to participate in a research endoscopy programme.

One study (da Rocha et al., 2008) has reviewed the endoscopic records of 101 patients who underwent oesophagectomy for end stage achalasia related to Chaga disease. In this study, endoscopic follow up was routine and the overall incidence of neo-Barrett's remained over 30% as seen in the present study.

The strength of the present study is that it is the largest to prospectively evaluate the incidence of neo-Barrett's in a relatively unselected patient population. Follow up was less complete than in the study of achalasia patients by da Rocha as one might expect given the cohort of more elderly patients with predominantly malignant diagnoses. These patients are, however more representative of those undergoing surgery in developed countries. The majority of patients underwent evaluation as part of a routine follow up programme rather than purely for the investigation of symptoms or having volunteered specifically to take part in a research project. Whilst there were exclusions of patients with known metastatic disease and those who were too frail for endoscopy to be clinically inappropriate, the inclusion criteria remain some of the broadest used in this type of study and are probably as encompassing as is practically and ethically possible.

The high incidence of neo-Barrett's occurring after subtotal oesophagectomy is likely to be related to reflux disease. Following surgery and reconstruction with a gastric conduit many patients experience profound duodeno-gastro-oesophageal reflux. In separate studies, Dresner and Öberg undertook manometric and 24 hour pH and bilirubin monitoring of post oesophagectomy patients (Dresner et al., 2003, Oberg et al., 2002). Dresner demonstrated

abnormal oesophageal exposure to both acid and bilirubin in 25 of 30 patients (83%). Öberg similarly demonstrated abnormal acid exposure in 25 of 32 patients (78%) and abnormal bilirubin exposure in 12 of 32 patients (38%). In both studies there was a significant association between the finding of pathological duodeno-gastro-oesophageal reflux and the presence of columnar metaplasia within the remnant oesophagus. This finding is not universal however and O'Riordan (2004), using similar methods reported that there was no significant association between the presence of pathological reflux and the presence of columnar metaplasia. The reason for this difference is unclear and considering the small number of patients involved it may well represent a statistical anomaly. Indeed this study did report a 50% incidence of columnar metaplasia in a sample of 48 patients and abnormal acid and bile reflux in 63% and 80% of a subgroup in which this was tested.

It has long been recognised that reflux disease is critical to the development of Barrett's oesophagus (Bremner et al., 1978). In order for acid to damage cells it is necessary for the H+ ions to penetrate the cell membrane. In the case of oesophageal epithelial cells, this membrane is somewhat resistant to this process and it is only when the luminal acidity is sufficient to damage intracellular junction that H+ ions are able to enter the cell in sufficient numbers to cause damage (Carney et al., 1981). Once H+ ions have entered the cell they cause inflammation and in severe cases, cell death by necrosis. When this occurs over a large area, this process leads to denuded, ulcerated areas of oesophagus.

Following oesophageal damage there is a process of repair and adaptation. It appears that conditions within the oesophageal lumen during this process influence the type of epithelium which regenerates. In the canine model, iatrogenic mucosal defects are repaired with squamous epithelium in the absence of significant ongoing reflux. When animals have excessive amounts of acid reflux induced this repair is with a metaplastic columnar epithelium (Bremner et al., 1978). It is thought that columnar epithelium is more resistant to further damage but the mechanism by which it is induced is poorly understood.

The inflammation induced by ongoing reflux is thought to have an important role in the induction of metaplasia. It has been hypothesised that this exerts its effect via the induction of transcription factors or the activation of developmental signalling pathways (Wang and Souza, 2011).

Transcription factors are proteins which bind to specific DNA sequences and control the rate of production of the protein products of these sequences. One example of a transcription factor potentially implicated in the development of Barrett's oesophagus is caudal related homeobox 2 (CDX2). Induction of this transcription factor appears to lead to squamous cells becoming more intestine-like, forming crypt like structures as seen elsewhere in the GI tract and expressing genes typical of intestinal cells. In human oesophageal squamous cells exposure to a combination of acid and bile salts increases CDX2 expression providing a potential step in the conversion to a columnar epithelium (Liu et al., 2006b, Hu et al., 2007).

Cell signalling pathways control basic cellular activities and allow cells to perceive and respond to their environment including by adapting following tissue injury. It is hypothesised that oesophageal damage and ongoing inflammation, may in some way, activate in oesophageal cells the signalling pathways normally involved in developing and maintaining the intestine (Wang and Souza, 2011). The activation of these pathways might, in turn, lead to the development of the intestinal-like epithelium seen in Barrett's oesophagus.

It appears that these processes are not solely dependent on the reflux of acid and that duodenal contents, in the form of bile acids and bile salts also have a role. Barrett's type epithelium has been reported following total gastrectomy and in a rat model with a duodeno-oesophageal anastomosis, both situations where one would expect acid reflux to be eliminated (Lillemoe et al., 1982, Meyer et al., 1979b). In the post-oesophagectomy setting there is gross reflux of both acid and duodenal contents, particularly when a pyloroplasty has been undertaken to aid gastric drainage. The high incidence of neo-Barrett's observed in the present study and other similar studies therefore may be related to the synergistic effects of the ongoing reflux of both acid and bile.

Gastro-oesophageal reflux disease is estimated to affect 10-20% of the Western population on a weekly basis (Dent et al., 2005), yet the estimated prevalence of Barrett's oesophagus is only 1-2%. Why some patients develop oesophagitis in response to reflux and others develop Barrett's or adenocarcinoma has never been adequately explained

Three distinct types of columnar epithelium have been noted in the oesophagus (Paull et al., 1976) and it is proposed that all of these are metaplastic and should be included in the definition of Barrett's oesophagus (British Society of Gastroenterology, 2005). This viewpoint is not universal and remains a source of controversy. The present study, where all three types of columnar epithelium were noted in biopsies from the oesophageal remnant provides important evidence to support the inclusion of all three in the definition of Barrett's oesophagus.

Many authors argue that only columnar mucosa with intestinal metaplasia represents true Barrett's oesophagus, associated with an increased risk of oesophageal adenocarcinoma (Wang and Sampliner, 2008). It has been claimed that a short segment of cardiac type mucosa is a normal finding at the gastro-oesophageal junction and that a normal oesophagus can be lined by cardiac mucosa in the distal 2cm (Hayward, 1961). The finding of cardiac type mucosa in the oesophageal remnant in this study and others provides clear evidence that this is a metaplastic epithelium as the whole gastric cardia and distal oesophagus is resected during surgery. Further support for this hypothesis comes from autopsy studies suggesting that the extent of cardiac mucosa increases with age (Ormsby et al., 2000).

A major difficulty in determining the normal histology of the gastro-oesophageal junction and distal oesophagus is the problem of accurately identifying the location from which endoscopic biopsies have been taken. One can only claim that Barrett's oesophagus is a spectrum of different types of columnar epithelium if it is certain that biopsies of all types have been taken from the oesophagus and do not represent inadvertent sampling of the gastric cardia or proximal stomach. Determining the location of the anatomical gastro-oesophageal junction in patients who have not undergone surgery can be

extremely difficult, especially in the presence of a hiatus hernia. The most commonly used definition in the West is the proximal extent of the gastric folds but this is dependent on the degree of insufflation of the oesophagus by the endoscopist.

In this study the site of the surgical anastomosis, representing the new oesophago-gastric junction, was clearly visible as a ring-like structure and in many cases, surgical staples were also visible. This allowed the endoscopist to locate biopsies with a degree of confidence not possible in patients who have not undergone surgery. It is far less likely, in this situation, that biopsies were inadvertently taken from the stomach and the finding of all three types of columnar epithelium strongly suggests that these can all be part of a spectrum of metaplastic change in the oesophagus. In the seminal study where the three types of oesophageal columnar epithelium were first described (Paull et al., 1976), manometric testing was used to ensure that biopsy material was obtained from above the lower oesophageal sphincter. The present study usefully employs an easily visible surgical anastomosis to achieve this result.

Several other studies employing endoscopic and histological evaluation in post-oesophagectomy patients have reported a range of histological subtypes in the oesophageal remnant. Some authors report the histological findings simply as columnar epithelium with or without specialised intestinal metaplasia but others list them according to either Paull's criteria or the modification of this system suggested by Chandrasoma (2000). As in the present study, Oberg (2002), D'Journo (2009) and Hamilton (1977) have all reported cardiac, fundic and specialised intestinal metaplasia columnar metaplasia in post-oesophagectomy patients. In contrast Dresner (2003), Lord (2004) and Peitz (2004b) have described only the cardiac or specialised intestinal metaplasia subtypes occurring. All three of these studies recognise the 3 subtypes described by Paull and state that they classified their samples according to these criteria suggesting that the difference is not due to the definitions used. The reason why these studies failed to find any examples of body or fundic type mucosa in contrast to the findings of the above listed and present studies is unclear. In the present study this type of mucosa accounted for 17 of 45 cases of columnar epithelium, in the study by Oberg it accounted for 3 of 15 cases and in

D'Journo's study it accounted for 13 of 42 cases, it is therefore not an infrequent finding in these studies.

Given the small number of cases of neo-Barrett's in the studies by Lord and Peitz (10 cases in each series) it is possible that the absence of any cases of body or fundic type mucosa is due to chance. The difference between the findings of the present study and that by Dresner et al with regards to this type of epithelium is particularly difficult to account for, given that these studies were conducted in the same unit, using similar methods and definitions with the main differences being the larger size and less selected nature of the cohort in the present study.

This specialised gastric type of epithelium, (also described as body or fundic type) most closely resembles the mucosa of the stomach to which the oesophagus has been anastomosed. The finding of this type could therefore represent proximal migration of this epithelium, metaplastic transformation of the native oesophageal mucosa or a sampling error with biopsies taken from the proximal gastric remnant. This latter explanation is somewhat difficult to accept as a sole explanation given that it has been reported in reasonable numbers of cases by multiple investigators, at different sites, each of whom describe being able to confidently located their biopsies above the anastomosis.

Results from this study effectively confirm that all three subtypes of columnar epithelium can develop in the previously squamous lined oesophagus and that all three types represent true metaplasia rather than sampling error or physiological columnar epithelium.

The post oesophagectomy model of the development of Barrett's is unique in that it allows the timescale over which metaplasia develops to be evaluated. Data from the present study and other similar studies suggests that Barrett's oesophagus can develop much more quickly in response to reflux disease than was originally thought. Sporadic Barrett's oesophagus is most commonly diagnosed in patients in their seventh decade of life suggesting that many years of reflux damage was required to induce metaplasia.

Cohort studies of patients with GORD have shown a significant association between the duration of reflux symptoms and the presence of sporadic Barrett's oesophagus. Locke and colleagues (1999) studied a series of 1011 patients scheduled for endoscopy for a variety of indications. Patients were asked to complete a validated symptom questionnaire, the results of which, along with the endoscopic findings were used to compile logistic regression models for conditions including Barrett's oesophagus. In this study duration of acid regurgitation was significantly associated with the presence of Barrett's. Those with a history of regurgitation for less than a year had 1.4 times the risk of Barrett's compared to those with no history of regurgitation. This risk rose to 2.7 times for those with a history between 1 and 5 years and 5.5 times baseline risk for those with symptoms dating back more than 5 years.

An association between duration of reflux symptoms and Barrett's oesophagus was also reported by Kulig et al. (2004). In this large prospective cohort study over 6000 patients completed symptom questionnaires and underwent endoscopic evaluation. Patients found to have Barrett's oesophagus were significantly more likely to report duration of reflux symptoms greater than five years compared to patients with erosive or non-erosive reflux disease. Gatenby and colleagues (2009) studied a cohort of over 1000 patients with known Barrett's oesophagus and found the median duration from the onset of symptoms to the diagnosis of Barrett's oesophagus without intestinal metaplasia to be only 2.6 years and for Barrett's oesophagus with intestinal metaplasia to be 5 years suggesting that metaplasia may develop more quickly than previously recognised and that there may be progression between subtypes. The problem with all cohort studies of this type is that they rely on the notoriously unreliable ability of a subject to recall their symptom history accurately.

Prior to oesophagectomy the cervical oesophagus is unlikely to have been exposed to significant amounts of duodeno-gastro-oesophageal reflux. Dual probe oesophageal pH monitoring has shown acid exposure in the proximal oesophagus to occur in less than 1% of a 24 hour period (Dobhan and Castell, 1993), sharply contrasting with the significant acid exposure seen following oesophagectomy. It is therefore proposed that the date of surgery represents a

baseline start date for any reflux related damage. The difficulties associated with using symptom history to determine the duration of reflux are therefore avoided.

In the present study the median post-operative period was significantly greater in patients with confirmed neo-Barrett's compared to those with no evidence of neo-Barrett's, 5.72 yrs vs 2.21 ($p=<0.001$). The earliest case of neo-Barrett's detected in this study occurred only 9 months following surgery. This data from post surgical patients clearly suggests that columnar metaplasia can occur within a few years of the onset of reflux and decades of damage are not required. Whether this rapid development is unique to neo-Barrett's, where patients experience particularly severe reflux is unclear but this finding certainly suggests that columnar metaplasia can develop more rapidly than was previously believed.

Other studies evaluating the time to develop neo-Barrett's have reported mixed results. O'Riordan et al. (2004) reported 24 cases of columnar metaplasia occurring in the oesophageal remnant, with the incidence rising from 36% at one year to 60% three years after surgery. Da Rocha et al. (2008) employed regular endoscopic evaluation in a follow up study of 101 patients who underwent surgery for end stage achalasia. The mean follow up period for patients in this series, 10.5 years is much longer than that for other series involving patients undergoing surgery predominantly for malignant disease. Again a steady increase in neo-Barrett's is reported with incidence rates of 11% at 5years, 30% for patients 5-10 years following surgery and 58% for patients followed up for more than 10 years following surgery. Whilst these patients underwent surgery for non-neoplastic disease they effectively underwent the same type of procedure and reconstruction as patients undergoing surgery for cancer. Post operatively they should therefore be just as susceptible to reflux as other patients and this would explain the similar incidence of neo-Barrett's observed in the cohort.

Oberg and colleagues (2002) report 15 cases of histologically confirmed columnar metaplasia in the oesophageal remnant at a median follow up of 4.9 years (range 3 – 10.4 years). In this study there was no evidence of the

prevalence of metaplasia increasing with time. The earliest endoscopy in this series occurred 3 years after surgery suggesting that when neo-Barrett's develops it tends to do so within the first 2-3 years following surgery. In the present study the prevalence of neo-Barrett's increased from 11% for patients 0-2 years following surgery to 43% for patients 2-4 years following surgery. The prevalence then remained relatively stable until patients got beyond 8 years post surgery. It is possible that there is a subgroup of patients who are pre-disposed to develop neo-Barrett' and do so within the first few years whereas other patients without this pre-disposition only develop metaplasia following a much more prolonged period of reflux associated injury.

There is some evidence to suggest that Barrett's with specialised intestinal metaplasia may be a more advanced form of the condition. In the present study there was a significant difference in the time that had elapsed between surgery and endoscopy in patients who had columnar metaplasia with specialised intestinal metaplasia (SIM) compared to those without (8.1 years vs 4.8 years, $p = 0.025$). This association between the presence of SIM and time from surgery has been reported elsewhere in smaller studies (Dresner et al., 2003, O'Riordan et al., 2004, Oberg et al., 2002). In addition progression between subtypes has been reported in individual patients. Dresner (2003) reports seven patients who initially had cardiac type metaplasia but at a subsequent endoscopy were found to have columnar metaplasia with SIM and D'Journo (2009) reports four patients who progressed from gastric type to cardiac metaplasia and 8 patients who progressed from cardiac type to SIM. No progression between subtypes was observed in the present study but only 15 patients with confirmed neo-Barrett's underwent a second endoscopy during the study period and the median time elapsed before the second endoscopy was only 12 months so this lack of progression is perhaps not surprising.

It is well recognised that the absence of SIM in Barrett's oesophagus may represent a sampling error rather than a true absence. The yield of SIM decreases as the segment of Barrett's shortens and the number of biopsies decreases and it has been estimated that a minimum of 8 biopsies is required to provide an adequate assessment (Harrison R et al., 2007). No study describes taking anywhere near this number of biopsies in post oesophagectomy patients

and sampling error may therefore partly explain the relatively low incidence of SIM. This is also likely to be the explanation for the absence of SIM in follow up biopsies in two patients in the present study who had SIM on initial biopsies. Sampling error due to small biopsy numbers is however, unlikely to contribute to the increasing incidence of SIM over time. There is no evidence to suggest that greater numbers of biopsies are taken over time, the majority of investigators having taken small numbers of biopsies according to a protocol in all patients as occurred in this study.

The consistent finding that the SIM subtype of metaplasia is found in patients who are endoscoped later and the progression in individual patients reported in some series provide compelling evidence that the development of Barrett's oesophagus is a stepwise process. The initial step is conversion to a non-intestinalised epithelium with subsequent progression to the classical specialised intestinal metaplasia (figure 3.10). The time frame for this progression appears to be highly variable with some individuals in the present study demonstrating no columnar metaplasia of any type more than 10 years after surgery and a small number developing SIM in around a year. Whether the two non-SIM subtypes represent successive steps or simply two variations of the initial step is unclear. The present study suggests that they are found at similar times following surgery but progression from gastric type to cardiac type has been reported elsewhere (D'journo et al., 2009).

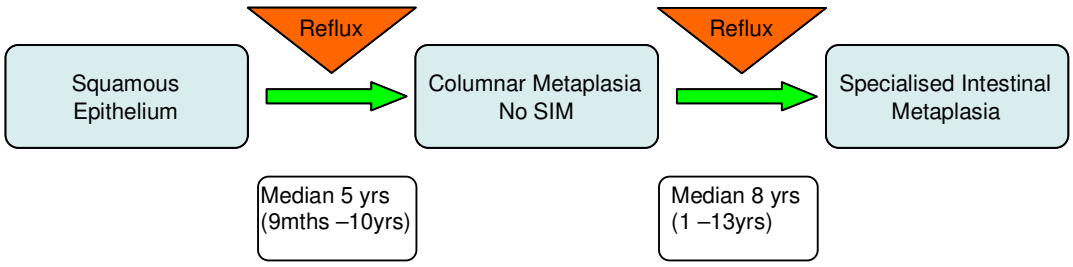


Figure 3.10 – Proposed mechanism for the development of Neo-Barrett's

Given the lack of progression between histological subtypes in the present study one could argue for a different model where there is development of different types of epithelium at different times without progression (figure 3.11). This would not however, be in keeping with other studies which have reported progression in individuals. Given that the present study included only a small number of patients with neo-Barrett's who underwent a second endoscopy and that median time between first and second endoscopy was only 12 months it does not provide good evidence against progression between histological subtypes and this model should be regarded as the less favoured one.

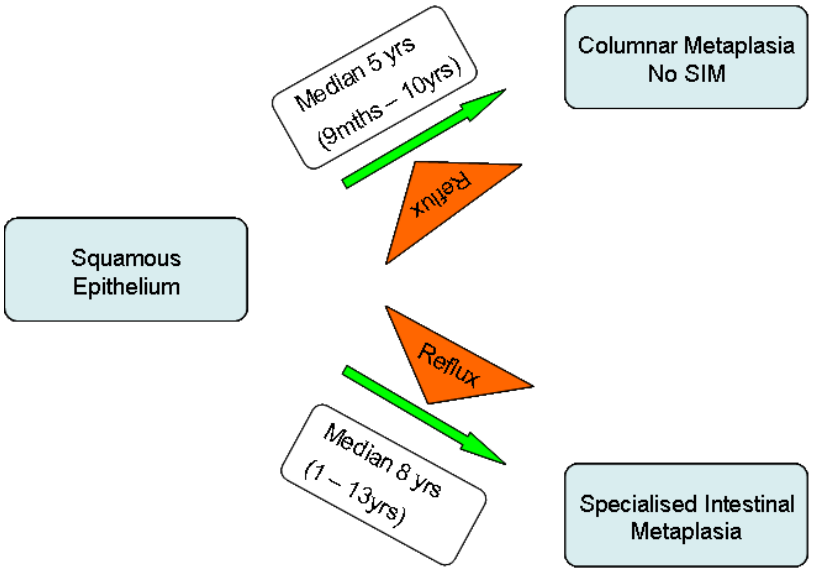


Figure 3.11: Alternative proposed mechanism for the development of Neo-Barrett's

In the present study the median length of Barrett's segment was relatively short at only 1.5cm. There was no evidence to suggest that segment length increased over time for the 15 patients who underwent more than one endoscopy. Unfortunately the median time between the first and second endoscopy was only 12 months and no comment can therefore be made on the long term stability of the segment length. Cameron and Lomboy (1992) suggested that when Barrett's oesophagus develops, the columnar segment

forms fairly rapidly and there is subsequently little increase in the segment length. They found that mean Barrett's segment length was similar for patients of all age groups studied and that there was no significant change in segment length for a subgroup of 21 patients with Barrett's oesophagus followed up for a mean of 7 years. Dresner et al. (2003) also found the area of columnar epithelium to be stable in post-oesophagectomy patients. Oberg (2002) describes a single patient with an increase in segment length from 1cm to 2cm between endoscopies at 13 and 52 months. Differences in segment measurements can vary according to observer and patient movements including retching and inspiration and a difference of 1cm is certainly within the margin of error for this type of measurement. Overall evidence from post-oesophagectomy patients is very limited but the few studies which have described segment length appear to support the hypothesis that Barrett's segments rapidly grow to their full length with little subsequent progression.

There are no clear predisposing factors for the development of neo-Barrett's following subtotal oesophagectomy. It has been proposed that pre-operative Barrett's oesophagus or adenocarcinoma might be important, as might surgical technique and demographic factors implicated in sporadic Barrett's such as male gender (D'journo et al., 2009, Dresner et al., 2003, O'Riordan et al., 2004) but consistent, high quality evidence is lacking.

In the present study neo-Barrett's was observed to develop following surgery for indications other than adenocarcinoma and high grade dysplasia in Barrett's. This finding confirms that neo-Barrett's represents *de novo* metaplasia rather than recurrent or residual disease. The incidence of neo-Barrett's was higher in patients who underwent surgery for disease on the Barrett's dysplasia/adenocarcinoma spectrum compared to others and in those with a pre-operative diagnosis of Barrett's compared to those without but neither difference was statistically significant. In common with this study, smaller studies by both Dresner and O'Riordan have reported no significant association between pre-operative histology and the development of neo-Barrett's. In contrast Oberg found that patients with a previous history of sporadic Barrett's were significantly more prone to developing neo-Barrett's (69% vs 25%, p=0.032) (Dresner et al., 2003, O'Riordan et al., 2004, Oberg et al., 2002).

D'Journo and colleagues (2009) found an association between a history of Barrett's and the occurrence of neo-Barrett's but this failed to reach significance on multivariate analysis. These findings serve to highlight the difficulties in defining significant risk factors in what are always going to be relatively small groups of patients.

Whilst statistical proof and a full understanding of pre-disposing factors remains elusive it is still possible to draw some useful conclusions from the present study. The presence of neo-Barrett's in patients with no previous history of Barrett's implies that these individuals do not have inherent infallible protective factors. This data suggests that any protective genetic or environmental factors against the development of metaplasia can be overwhelmed in the context of severe acid and bile reflux such as that which occurs following oesophagectomy and reconstruction with a gastric conduit. Patients with no previous history of Barrett's may simply never have experienced the 'critical' amount of reflux required to trigger metaplastic transformation prior to surgery. The present study did not attempt to assess pre-operative reflux and this could be an interesting area for future study.

The incidence of neo-Barrett's was similar for men and women in this study (40% vs 31%, p=0.304). This is despite the fact that that only 44% of women underwent surgery for dysplastic Barrett's or adenocarcinoma compared to 88% of men which as discussed previously has been suggested as a pre-disposing factor in some series. In non-surgical groups the incidence of Barrett's has consistently been found to be higher in men with ratios of 2:1 described in an autopsy study in the United States (Cameron and Lomboy, 1992) and 1.7:1 in the United Kingdom Barrett's registry (Caygill et al., 2003). Again the roughly equivalent incidence of neo-Barrett's in men and women following surgery suggests that severe reflux is the critical factor in the development of metaplasia and outweighs any potential protective gender influences.

In patients who have not undergone surgery there appears to be a correlation between symptomatic gastro-oesophageal reflux disease and the development of Barrett's oesophagus. In the present study 66% of patients had either intermittent or ongoing reflux symptoms but there was no statistically significant

association between this symptomatic reflux and the development of neo-Barrett's. Pathological gastro-oesophageal reflux has been shown to be almost universal following subtotal oesophagectomy and reconstruction with a gastric conduit (Dresner et al., 2003, O'Riordan et al., 2004, Oberg et al., 2002). Where a pyloroplasty has been undertaken the refluxate generally contains abnormally high levels of bile which has also been implicated as a causative factor in the development of columnar metaplasia. It is therefore likely that even patients who did not report symptomatic reflux in this study were likely to experience pathological levels of reflux, albeit asymptomatically and this may explain the absence of an association between symptomatic reflux and neo-Barrett's. Many patients in the present study were taking proton pump inhibitor medication which is likely to have affected their experience of reflux. The medication may have diminished or abolished the typical symptoms of GORD recognised by patients and led to a negative response when questioned.

A potential weakness of the present study is the failure to use a validated questionnaire to assess reflux. This would not have overcome all of the problems outlined above but might have improved the accuracy of symptom assessment. Unfortunately even detailed questionnaires rely on the ability of patients to recall a symptom history. Such questionnaires can be very useful in assessing current or recent symptoms but assessing reflux since surgery requires recalling symptoms over a number of years in some cases. Only prospective collection of this data in all patients might significantly improve the quality of this data.

O'Riordan and colleagues (2004) did employ a more robust validated questionnaire and demonstrated an association between reflux symptoms and the degree of supine acid reflux in post oesophagectomy patients suggesting that symptoms may be a marker of reflux (O'Riordan et al., 2004). Unfortunately some patients in this study with low symptom scores had high levels of bile reflux and some patients with low symptom scores also had neo-Barrett's. Taken with the findings of the present study it can be concluded that reflux symptoms provide neither an accurate assessment of the presence of duodeno-gastro-oesophageal reflux nor an assessment of the risk of columnar metaplasia.

Several groups have suggested that the type of surgical anastomosis is important in controlling reflux and reflux related damage in the oesophageal remnant (De Leyn et al., 1992, D'journo et al., 2009, Shibuya et al., 2003).

Although a standard surgical approach was used in all patients in this study there was variability in the measured distance from the incisors to the surgical anastomosis at the time of endoscopy, recorded values being between 18 and 34cm (median 24cm). Traditional surgical teaching advises that constructing the anastomosis more proximally reduces the severity of reflux. With the exception of the patient with an anastomosis measured at 34 cm this variability most likely reflects variations in the height and build of patients and in the extent of dissection related to the site of the primary tumour. In the case of the patient with an anastomosis at 34cm one must wonder whether the surgical approach was as described or if there were particular intra-operative difficulties. There is no evidence in the present study that it is possible to reduce the risk of neo-Barrett's by siting the oesophago-gastric anastomosis as high as possible using the trans-thoracic approach.

The best evidence for the importance of surgical technique comes from a recent paper by D'Journo et al. (2009) comparing symptoms and endoscopic findings in 84 oesophagectomy patients, 36 of whom had an anastomosis in the right upper chest and 48 of whom had an anastomosis in the left neck. Patients with a right intra-thoracic anastomosis were more likely to experience reflux symptoms (81% vs 52%, p=0.007) and were more likely to have developed columnar metaplasia in the oesophageal remnant despite having a shorter median follow up period (66% vs 37%), this difference remained significant on multivariate analysis, p=0.018. Other studies have found the incidence of oesophagitis to be higher when an intra-thoracic anastomosis is employed compared to a cervical anastomosis (Shibuya et al., 2003, De Leyn et al., 1992).

In contrast to clinical studies which suggest that a cervical anastomosis may decrease reflux, the only study which has used ambulatory pH monitoring suggested that acid exposure in the oesophageal remnant is higher in patients with a cervical anastomosis.(Johansson et al., 1999) In this study, no patient

had undergone a pyloroplasty and no absolute or statistical values are quoted to support this finding making it difficult to draw conclusions from the findings. The absence of any relation between the presence of neo-Barrett's and the height of the anastomosis in the present study may simply reflect the fact that all patients had very similar surgical procedures. Differences in the height of the anastomosis having simply reflected the differences in patient build and the inevitable minor inaccuracies in measuring the distance from the incisors rather than a true significant difference in the anatomical position of the anastomosis.

The present study failed to identify any statistically significant predisposing factors for neo-Barrett's. It is likely that the origins of the condition are multi-factorial and might be revealed only by multivariate analysis of large numbers of patients. Whilst the present study is the largest of its kind, the numbers of patients remain too small and the number of potential pre-disposing factors too large for a multivariate analysis of these risk factors to be appropriate.

Neo-Barrett's oesophagus following subtotal oesophagectomy may have important implications for affected individuals. Should it have the same potential for malignant progression as sporadic Barrett's oesophagus this would need to be factored in to treatment decisions and follow up protocols.

In the present study there were no cases of neo-Barrett's oesophagus with dysplasia. There are case reports of dysplasia and adenocarcinoma occurring in the context of neo-Barrett's suggesting that it has some malignant potential but the numbers are small and the magnitude of the risk difficult to determine. There are 2 reported cases of adenocarcinoma occurring more than four decades after the original surgery, one from the Northern Oesophago-gastric cancer unit and one from the United States (Dunn et al., 2010, Lord et al., 2004). In both of these cases, surgery was undertaken for benign strictures occurring in childhood. Two cases of neo-Barrett's with high grade dysplasia have been reported following surgery for achalasia (da Rocha et al., 2008). In these cases the time from surgery to the development of dysplasia was shorter, 13 and 19 years respectively. In both cases there was progression to invasive adenocarcinoma within 2 years supporting the potential for progression along a

metaplasia-dysplasia-adenocarcinoma sequence as is seen in sporadic Barrett's oesophagus.

Median follow up in the present study at 3.6 years (range 0.3 – 13.2) was much shorter than would be required for cases of the above type to be detected. There is a single detailed case report describing a case of neo-Barrett's in a patient within the time that might have been observed in the present study (Gutschow et al., 2008). Dysplasia was detected 15 months after subtotal oesophagectomy for a Barrett's adenocarcinoma and by 28 months this had progressed to invasive adenocarcinoma. The original proximal resection margin representing the residual oesophagus is reported as being free from metaplasia but the reason why this individual should have developed such early dysplastic neo-Barrett's is unexplained. The absence of any cases of dysplasia in a cohort the size of the present study provides some reassurance that such cases are rare. With the majority of patients undergoing surgery in later life the numbers whose natural life expectancy is such that they might be at risk of malignant progression in neo-Barrett's is even smaller given that in all but one case it appears to take more than a decade to develop.

In summary, the present study has demonstrated that over a third of patients will develop neo-Barrett's in the oesophageal remnant following subtotal oesophagectomy and reconstruction with a gastric conduit. All three subtypes of columnar epithelium are confirmed as being metaplastic having developed in previously squamous epithelium. The findings of this study suggest that the first step in the development of Barrett's oesophagus is conversion to a non-intestinalised columnar epithelium with subsequent conversion to specialised intestinal metaplasia in the presence of ongoing reflux. The timescale over which this occurs is variable but appears to be measured in years. The presence of neo-Barrett's cannot be predicted on the basis of clinical symptoms, demographic details or the original indication for surgery.

Chapter 4: Cellular protein expression in metaplastic columnar epithelium in the remnant oesophagus following subtotal oesophagectomy

4.1 Introduction

Barrett's oesophagus is defined as the replacement of the normal stratified squamous epithelium of the oesophagus with a metaplastic columnar epithelium. Only columnar epithelium in which deep oesophageal glands are present is viewed as incontrovertible evidence of Barrett's oesophagus yet very few endoscopic biopsies demonstrate these features. The majority of biopsies show columnar epithelium with no deep oesophageal glands. In these cases, knowledge of the exact location from which the biopsies were taken is essential in confirming the diagnosis. Biopsies from the oesophagus, which demonstrate columnar epithelium, corroborate a diagnosis of Barrett's. It is however, important to recognise that the precise location of the gastro-oesophageal junction may be difficult to locate, particularly when a hiatus hernia is present. An endoscopist attempting to take a biopsy from just above the junction may easily and inadvertently sample the gastric cardia. In this case the biopsies would have a columnar epithelium but a diagnosis of Barrett's oesophagus would be inaccurate. Even where specialised intestinal metaplasia is present, it is unclear if this carries the same malignant potential when it originates in the gastric cardia as when it originates in the distal oesophagus in Barrett's.

.A diagnosis of Barrett's oesophagus can have significant implications for an individual. There can be elevated levels of anxiety and insurance premiums may be increased. Patients may be enrolled on a Barrett's oesophagus surveillance programme which commits them to repeated endoscopic evaluation. On a population basis, overdiagnosis of Barrett's oesophagus would have significant cost implications associated with unnecessary endoscopic surveillance. For these reasons there has been considerable interest in developing a more robust diagnostic test to confirm and endoscopic diagnosis of Barrett's.

Immunohistochemical techniques employ antibodies to visualise substances in tissue sections. Antibodies bind to a specific substance of interest if this is present within the cell or its membrane. The bound antibody can subsequently be visualised using one of a variety of techniques. As different biomarkers are expressed by different tissues types, this technique can be used to identify tissues especially when conventional histology is inconclusive.

Immunohistochemistry is widely used to type poorly differentiated tumours according to their tissue of origin (Dennis et al., 2005).

Immunohistochemistry has been considered as a diagnostic tool for use in Barrett's oesophagus and numerous studies have tried to identify sensitive and specific markers. An ideal marker could be used to confirm the diagnosis and to differentiate metaplastic columnar epithelium of oesophageal origin from non-metaplastic proximal gastric epithelium (Ormsby et al., 1999). Several potential markers have been studied, the most widely reported of these being cytokeratins. The clinical role of this technique in the diagnosis of Barrett's oesophagus remains a source of controversy but there is increasing evidence on the presence and pattern of various markers within Barrett's tissue.

The post-oesophagectomy neo-Barrett's theory suggests that the columnar metaplasia seen following surgery is a good model for the development of Barrett's oesophagus. The aim of this study was to determine if the same cellular proteins seen in sporadic Barrett's oesophagus are present in post oesophagectomy neo-Barrett's thereby supporting its role as an accurate model.

4.1.1 Cytokeratin Immunostaining

Cytokeratins (CKs) are a family of structural proteins that, along with microfilaments and microtubules, are constituents of the cytoskeleton of epithelial cells. The cytoskeleton is responsible for maintaining the mechanical integrity of the cell. It is also important in cell division, motility and cell to cell contact. Cytokeratins are encoded for by a large family of genes but they each exhibit a similar structure with a central helix-rich rod and non-helical N and C terminal domains (Barak et al., 2004).

More than 20 different types of CK are known and their expression is highly variable depending on the type and location of the epithelium. Even within the gastrointestinal tract the cytokeratin expression pattern varies along its length. The mouth and oesophagus express CK6, a cytokeratin typically associated with stratified squamous epithelia (Chu and Weiss, 2002). CK20 is generally considered a marker of intestinal differentiation and is expressed by the surface

foveolar epithelium of the stomach and by the surface and crypt epithelium of the small intestine and colon. CK7 is associated with ductal differentiation and is expressed by the epithelia of bile and pancreatic ducts. In addition CK7 expression has been reported in the gastric cardia although it is consistently absent from the gastic body and antrum (Jovanovic et al., 2002).

In malignancy, the cytokeratin patterns associated with the original tissue type are usually maintained, a property which has allowed cytokeratins to be used as tumour markers (Chu and Weiss, 2002). As the expression of individual cytokeratins is not organ specific a panel of markers are usually assessed and evaluated along with the morphology of a haematoxylin and eosin stained section of a specimen.

In 1999 Ormsby and colleagues (1999) described a distinctive pattern of cytokeratin 7 and 20 staining in Barrett's oesophagus. This classical Barrett's pattern is outlined in figure 4.1.

CK7 Diffuse moderate to strong staining of both superficial and deep glands
CK20 Band-like staining of surface epithelium and superficial glands

Figure 4.1: Classical Barrett's cytokeratin (Ormsby et al., 1999)

This pattern was found to be present in 29/30 (94%) of resection specimens of long segment Barrett's oesophagus and 34/34 biopsies from long segment Barrett's oesophagus The authors defined long segment Barrett's oesophagus as being greater than 3cm in length. Histologically the definition required the presence of columnar epithelium with specialised intestinal metaplasia.

In this study Barrett's specimens from the oesophagus were compared to gastric specimens also demonstrating intestinal metaplasia. Endoscopic biopsies were obtained from the gastric cardia within 5mm of an apparently

normal squamo-columnar junction and gastrectomy specimens were used to obtain samples of gastric intestinal metaplasia originating at least 3cm distal to the gastro-oesophageal junction. The CK7/20 pattern described above was highly specific for Barrett's oesophagus as this pattern was not seen in any of the gastric samples. The exceptionally high specificity seen in this study has not been replicated elsewhere but other authors have confirmed the presence of this staining in the majority of cases of Barrett's oesophagus (deMeester et al., 2002, Glickman et al., 2001) and it was therefore felt to be a useful marker pattern to explore in neo-Barrett's.

4.1.2 Chromogranin A Immunostaining

Neuroendocrine cells are responsible for the uptake and release of neurotransmitters and neuropeptide hormones and are an integral part of the intestinal epithelium (Voutilainen et al., 2002). Neuroendocrine differentiation is well described in gastric adenocarcinomas where it may be associated with a slightly better prognosis and in colonic adenocarcinomas where the association with prognosis is uncertain (Rogers and Murphy, 1979) (Smith and Haggitt, 1984, Grabowski and Schindler, 2001). The presence of neuroendocrine differentiation within areas of Barrett's oesophagus has been reported by a number of authors (Koppert et al., 2004, Hamilton et al., 2000, Griffin and Sweeney, 1987, Jaskiewicz et al., 1994). The presence of these cells is notable because it suggests that Barrett's arises from a multipotential stem cell capable of differentiating into more than one cell type (Koppert et al., 2004).

Chromogranin A is regarded as a general marker of neuroendocrine differentiation. It is stored in the secretory granules of neuroendocrine cells and participates in vesicle aggregation , granulogenesis and hormone secretion (Koppert et al., 2004). Other markers of neuorendocrine differentiation include synaptophysin and neuron-specific enolase (NSE) but these are less widely studied in the oesophagus. There are no reports of the use of synaptophysin or NSE to detect neuroendocrine cells in Barrett's oesophagus and only one study describes the use of synaptophysin to detect neuroendocrine cells in oesophageal adenocarcinoma (Wang et al., 2006). Chromogranin A was therefore selected as the marker of neuroendocrine differentiation in this study. .

The present study sought to establish whether there was evidence of neuroendocrine differentiation within neo-Barrett's oesophagus similar to that reported in Barrett's oesophagus.

4.2 Methods

Inclusion criteria for the study were as follows:

1. Confirmed neo-Barrett's (endoscopic evidence of columnar epithelium above the surgical anastomosis with histological corroboration.

2. No evidence of Barrett's oesophagus at the proximal resection margin at the time of surgery i.e. residual Barrett's oesophagus.

3. Informed consent given by the patient either via a specific consent form for this study or via a generic consent form used in the endoscopy department for tissue to be used for research purposes.

4.2.1 Biopsy technique

As detailed in the previous chapter biopsies were taken from above the level of the surgical anastomosis. Histological analysis was undertaken as previously described and the columnar epithelium classified as one of three subtypes.

4.2.2 Immunohistochemistry Technique

Unstained 5μm tissue sections of formalin fixed paraffin-embedded tissue were cut according to the protocol described in appendix D. Staining was undertaken using the Ventana Benchmark XT automated staining system (Roche diagnostics, Sussex, UK), protocols used are outlined in appendices E and F. A primary antibody raised in rabbits was used for cytokeratin 20 (Ventana, Tucson, Arizona, USA), primary antibodies raised in mice were used for cytokeratin 7 and chromogranin A (Dako UK Ltd, Ely, Cambridgeshire). In all cases the ultraView Universal DAB detection kit (Ventana, Tucson, Arizona, USA) was used to visualise positive binding.

4.2.3 Analysis

Slides were evaluated by the author and any equivocal slides were reviewed by an expert gastrointestinal pathologist. Many patients had examples of more than one type of columnar epithelium within the biopsy samples. Where intestinal metaplasia was present, this was always assessed. In other cases

the most representative areas of the predominant histological subtype were scored.

Cytokeratins

CK 7 and 20 staining was evaluated for both the superficial and deep compartments (table 4.1).

Classification	Description
Diffuse	Staining of all representative columnar epithelium
Patchy	Staining of some, but not all representative columnar epithelium
Absent	No staining of representative columnar epithelium

Table 4.1: Scoring system for cytokeratin staining

Patients with band-like CK20 staining of the surface epithelium and superficial glands and diffuse moderate to strong staining of both superficial and deep glands as described by Ormsby were classified as demonstrating a classical Barrett's phenotype.

Chromogranin A

Staining for Chromogranin A utilised a semi-quantitive scoring system (table 4.2)

Score	Description
0	No staining
1+	Very rare staining, scattered single cells
2+	More frequent individual cells
3+	Small clusters of cells
4+	Nodule formation

Table 4.2: Semi-quantitive scoring system for Chromogranin A

4.2.4 Statistics

Fisher's exact test was used for comparisons of proportions. The Mann-Whitney U test was used to compare continuous data between groups with the Kruskal-Wallis test used for comparisons between multiple groups. Correlation was analysed using the Spearman correlation coefficient.

4.3 Results

4.3.1 Study population

The study population comprised a total of 37 patients, 31 males and 6 females. Median time following surgery was 6.6 years (range 1.0 – 13.2 years).

Thirty five patients were endoscoped prospectively by, or in the presence of the author. The remaining two patients were identified retrospectively and had clear documentation and picture evidence of columnar epithelium above the anastomosis from which biopsies had been taken.

All three subtypes of columnar epithelium were represented in the study as summarised in table 4.3.

Barrett's Subtype	Number of patients
A (specialised intestinal metaplasia)	12
B (body or fundic type)	16
C (cardiac type)	9

Table 4.3: Histological subtypes of patients included in the study

4.3.2 Cytokeratin 7 and 20 immunostaining

Fragments of normal squamous epithelium were frequently seen alongside columnar epithelium in the biopsy samples from individual patients. In all cases, areas of squamous epithelium were negative for both CK7 and CK20.

Cytokeratin 7

Of the two cytokeratins studied, CK 7 was the more widely expressed throughout the epithelium (figure 4.2). In the superficial compartment, diffuse staining was seen in 27 cases (73%) and patchy staining was seen in 9 cases (24%). One case of the type B subtype had no CK7 immunostaining in the superficial compartment. All cases demonstrated CK7 immunostaining in the deep compartment, in 27 cases (73%) this was diffuse and in 10 cases (27%) this was patchy. The case with no superficial staining demonstrated diffuse deep staining.

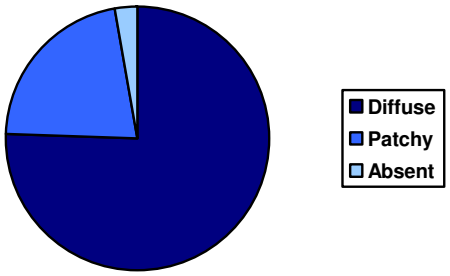


Figure 4.2a: Extent of CK7 staining of the superficial compartment

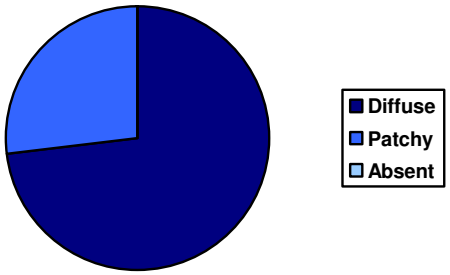


Figure 4.2b: Extent of CK7 staining of the deep compartment

Cytokeratin 20

Strong band-like superficial CK20 staining was seen in 36/37 cases (97%). In the remaining case there was patchy staining. In all cases CK20 staining was absent from the deep compartment (figure 4.3).

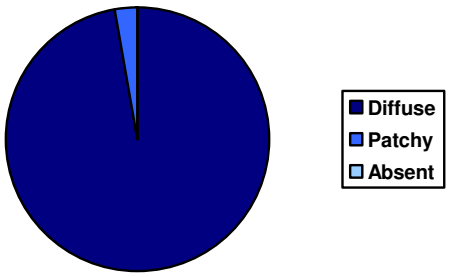


Figure 4.3a: Extent of CK20 staining of the superficial compartment

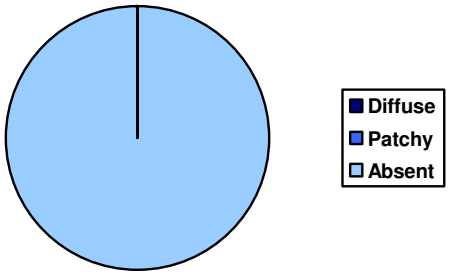


Figure 4.3b: Extent of CK20 staining of the deep compartment

Classical Barrett's Pattern Staining

The classical Barrett's CK7/20 staining pattern was seen in 23/37 cases (62%). The band-like staining of the surface epithelium and superficial glands for CK20 and the diffuse superficial and deep staining for CK7 are shown in figure 4.4.

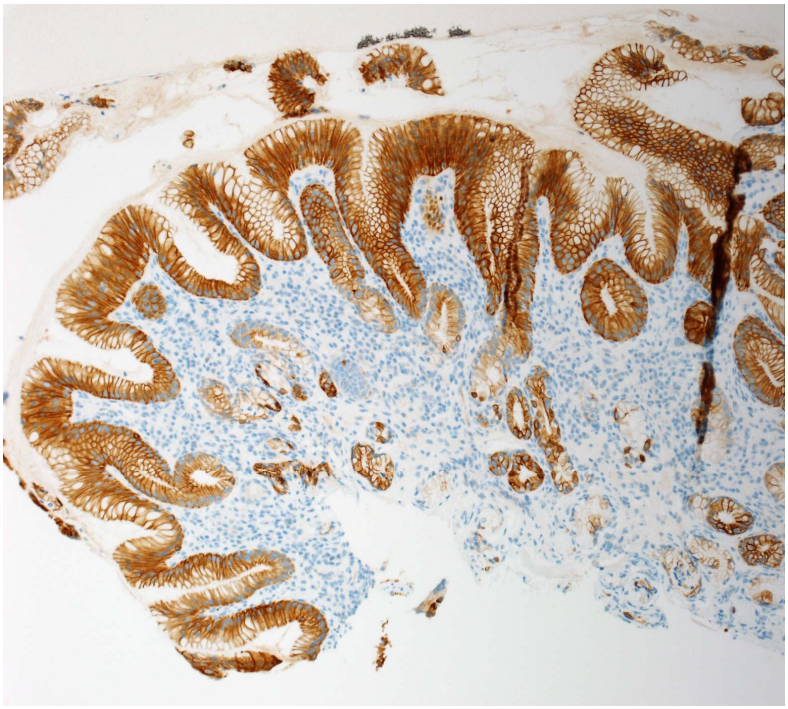


Figure 4.4a: CK7 - Diffuse staining of both superficial and deep glands

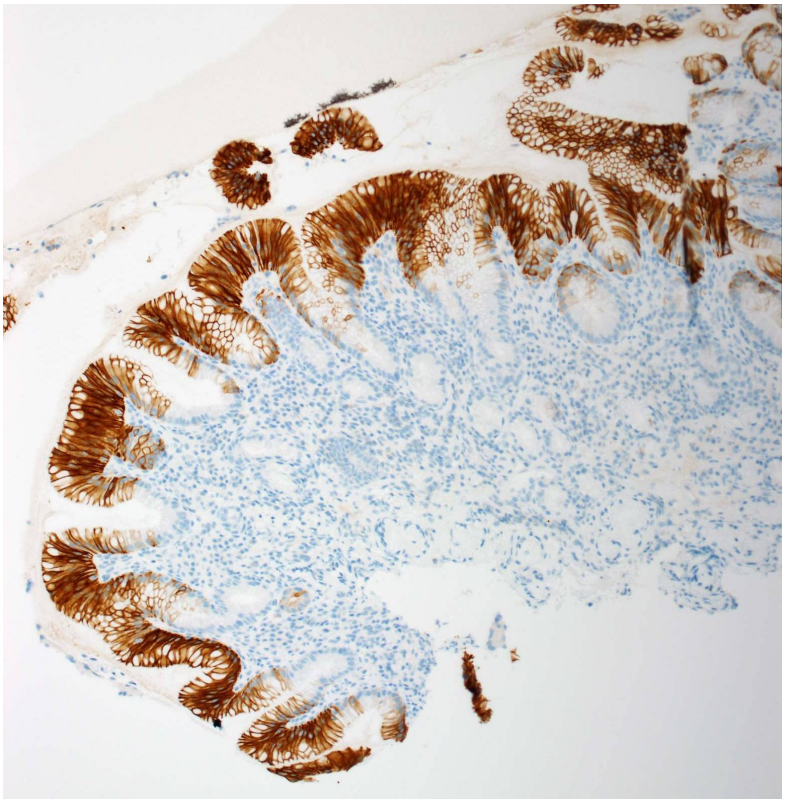


Figure 4.4b: CK20 – Band like superficial staining for CK20

The proportion of each histological subtype of columnar metaplasia which stained with a classical Barrett's pattern is shown in figure 4.5. The proportion of patients with this classical Barrett's type CK7/20 staining pattern was greater in the group with intestinal metaplasia (Type A) compared to those without intestinal metaplasia (Types B and C) but this difference failed to reach statistical significance (p=0.084).

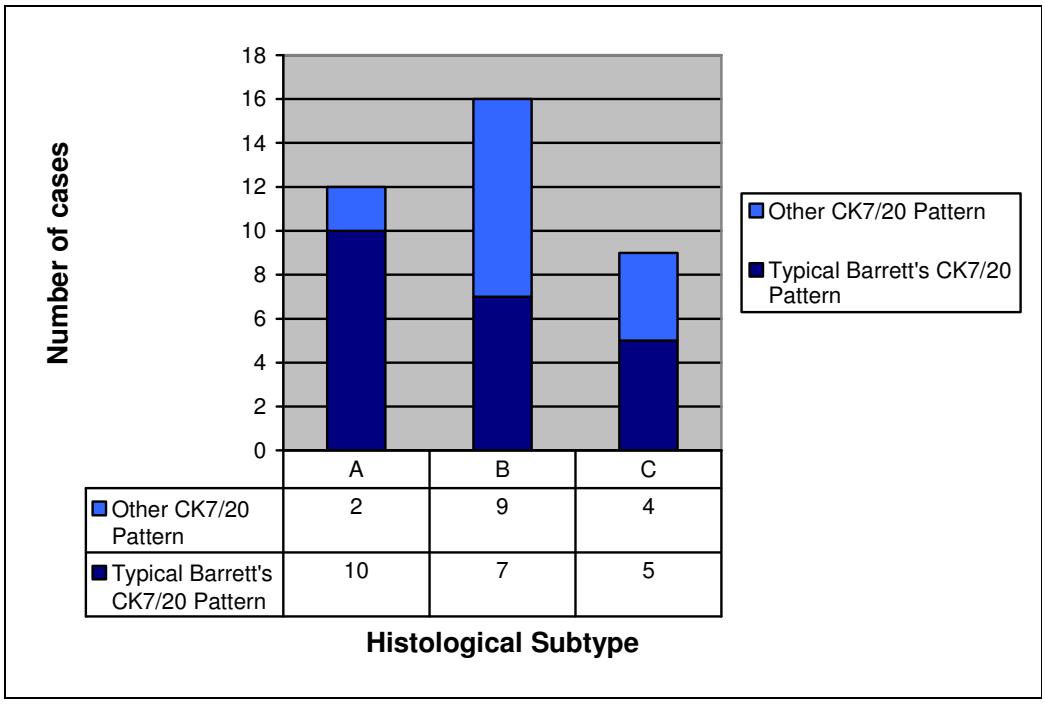


Figure 4.5: Typical Barrett's staining according to histological subtype

The median time that had elapsed between surgery and endoscopic biopsy was 7.8 years for patients with a classical Barrett's CK7/20 staining pattern and 5.1 years for those that did not demonstrate this pattern. This difference was not statistically significant (p=0.578) (figure 4.6).

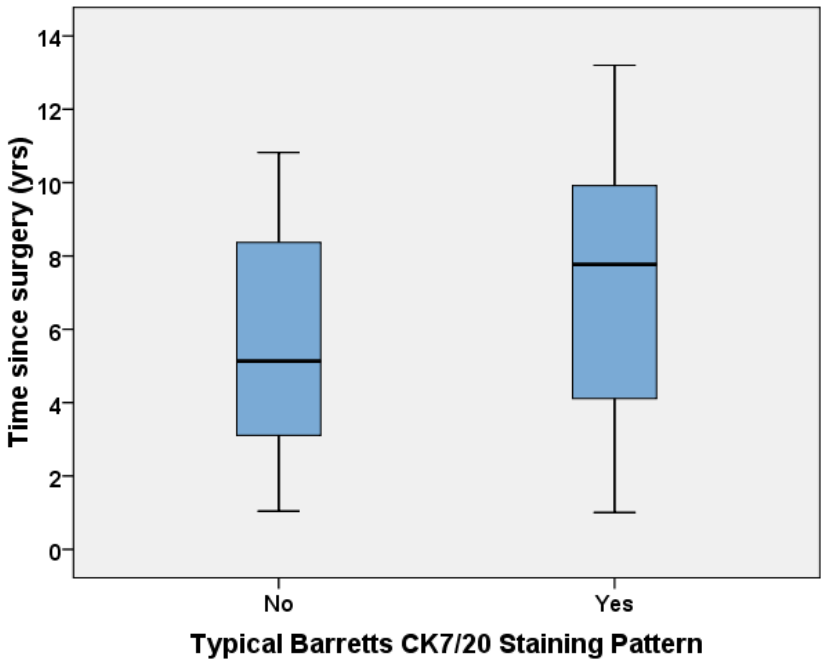


Figure 4.6: Box plot showing the association between time elapsed since surgery and the presence of a classical Barrett's CK7/20 staining pattern. There was no significant difference (p=0.340)

4.3.3 Chromogranin A immunostaining

All 37 neo-Barrett's samples demonstrated expression of chromogranin A to some extent. Table 4.4 shows the frequencies of the different staining intensities and examples of these staining intensities are shown in figure 4.7. The most frequent pattern of chromogranin A expression seen was that of rare, scattered single cells (1+).

Chromogranin A Score	Number of samples (%)
0	0
1+	22 (59%)
2+	7 (19%)
3+	8 (22%)
4+	0

Table 4.4: Staining intensities for Chromogranin A in neo-Barrett's samples

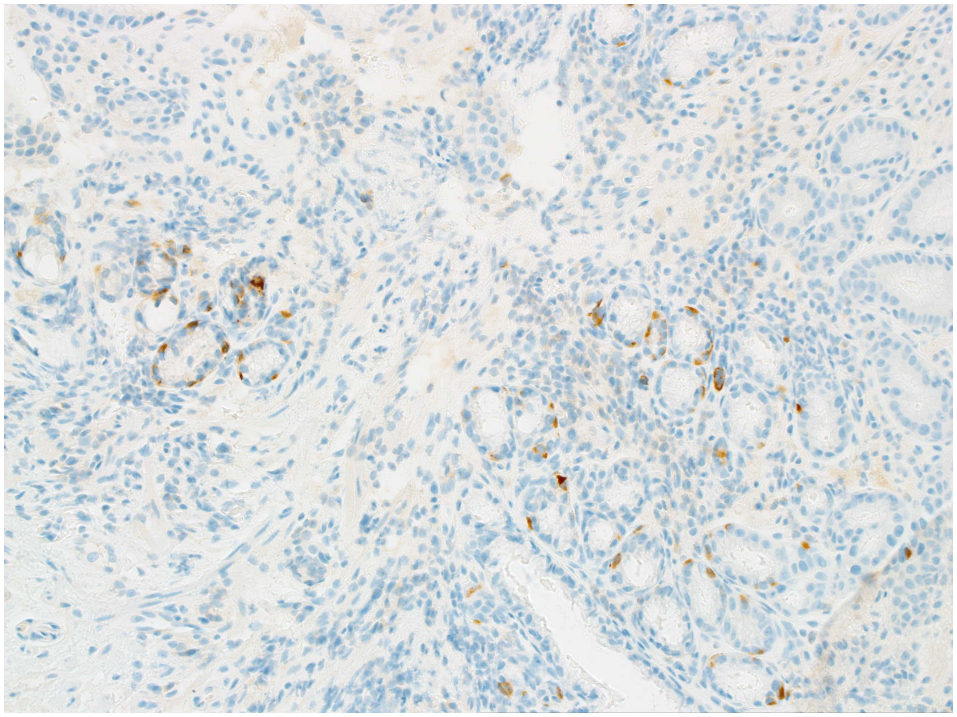


Figure 4.7a: An example of 1+ Chromogranin A staining with infrequent staining of single scattered cells

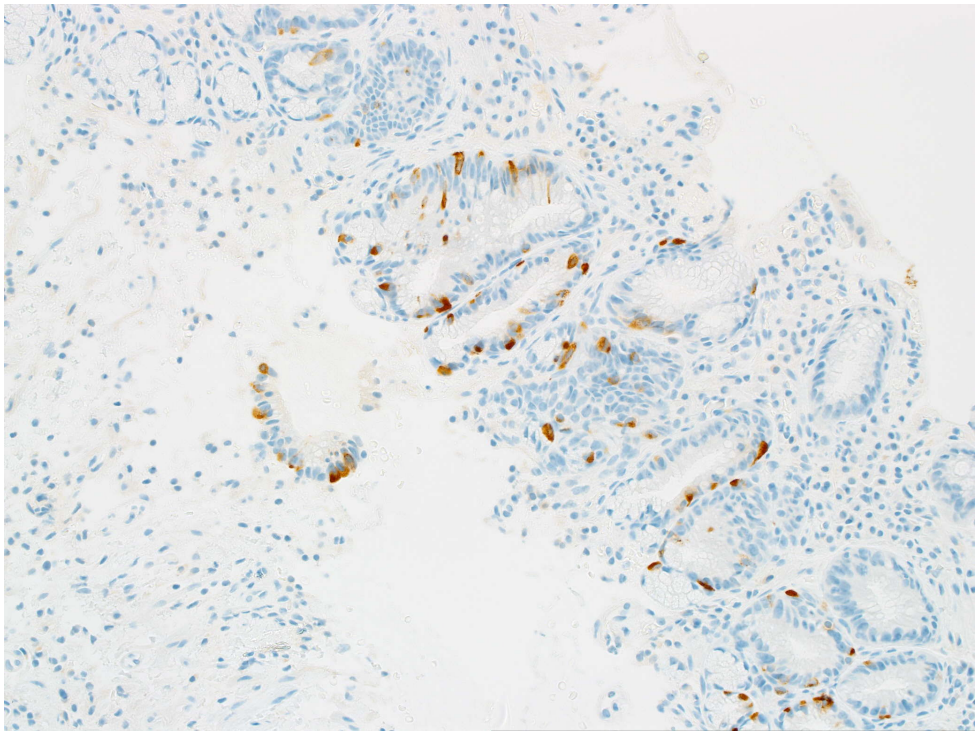


Figure 4.7b: An example of 2+ Chromogranin A staining with more frequent staining of individual cells

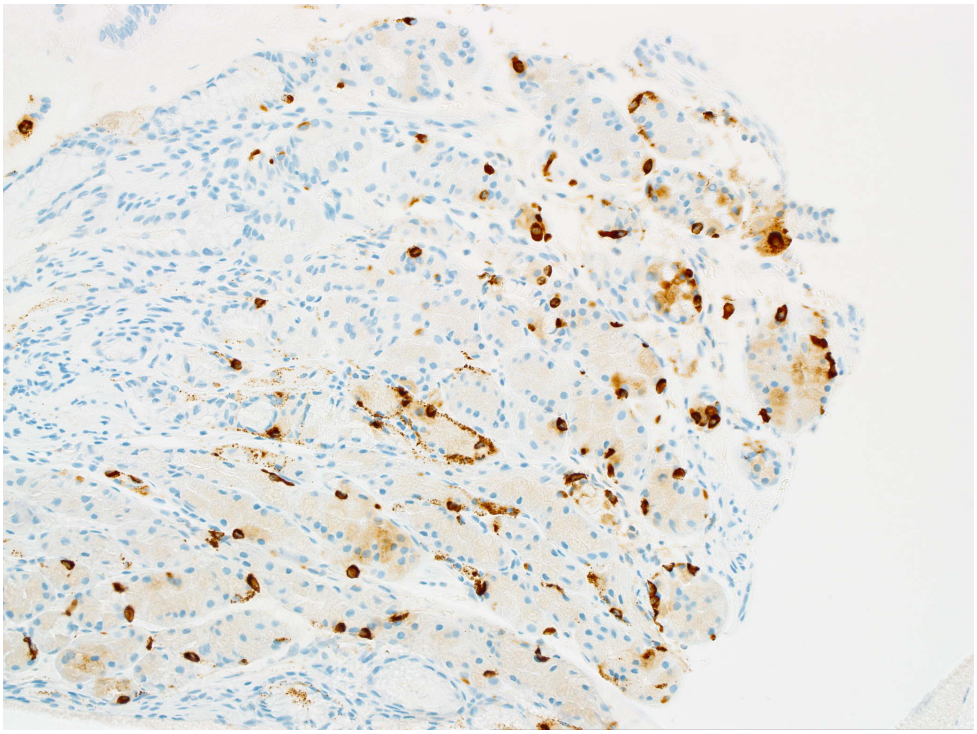


Figure 4.7c: An example of 3+ Chromogranin A staining with small clusters of positive cells

The Chromogranin A staining intensities for the three histological subtypes of columnar metaplasia are given in table 4.5. There was no significant difference in the staining intensity for the different subtypes (p=0.180)

Histological subtype	1+	2+	3+	Mean score
A (specialised intestinal metaplasia)	5	5	2	1.75
B (body or fundic type)	9	2	5	1.75
C (cardiac type)	8	0	1	1.22

Table 4.5: Chromogranin A staining intensity according to subtype of columnar epithelium

There was no correlation between the time since surgery and the Chromogranin A staining intensity (p=0.697) (figure 4.8).

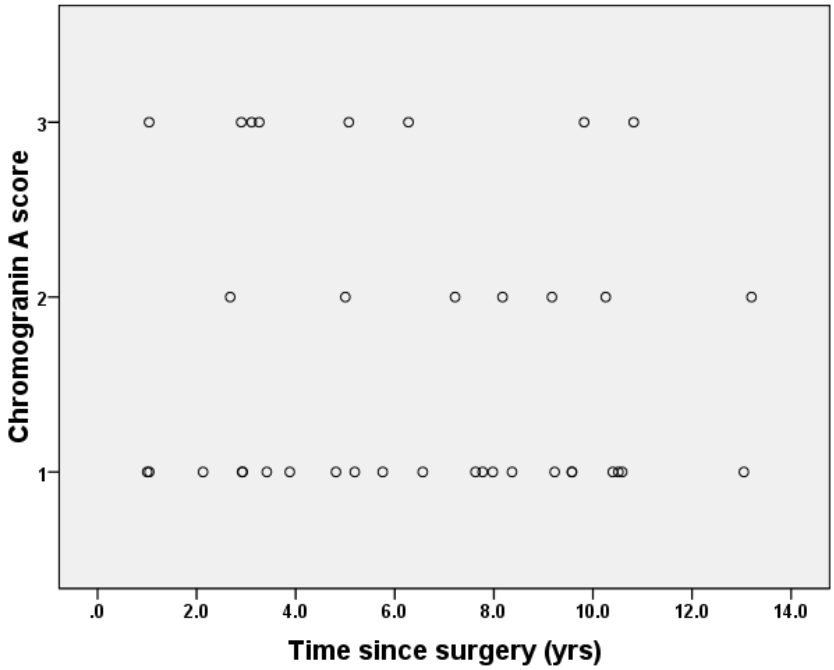


Figure 4.8: Scatter plot of time since surgery and Chromogranin A score

4.4 Discussion

The columnar metaplasia occurring in the oesophageal remnant following subtotal oesophagectomy has been proposed as a suitable human model in which to study the development of Barrett's oesophagus. For this model to hold true it is important to be certain that this mucosa is molecularly similar to Barrett's oesophagus in addition to being endoscopically and histologically similar.

The diagnosis of sporadic Barrett's oesophagus represents a significant clinical challenge, particularly in cases where only a short segment may be present. Few biopsies show the combination of deep oesophageal glands and columnar epithelium required to confirm the diagnosis of Barrett's oesophagus. In addition, specialised intestinal metaplasia, which is sometimes considered a hallmark of Barrett's, can occur in the gastric cardia and the clinical significance of this intestinal metaplasia of the cardia is uncertain. For these reasons much work has focused on finding molecular markers which are specific for Barrett's oesophagus. The most widely studied potential markers are cytokeratins 7 and 20.

A cytokeratin pattern that might be specific for Barrett's oesophagus was first described by Ormsby and colleagues (1999). They performed cytokeratin 7 and 20 staining on biopsy samples and surgical resection specimens and compared the cytokeratin staining pattern in patients with Barrett's oesophagus to those with intestinal metaplasia of the gastric cardia. The classical CK7/20 staining pattern of superficial CK20 staining and strong CK 7 staining for both superficial and deep glands was found to be present in 29 of 31 (94%) of oesophageal resection specimens and all of 34 oesophageal biopsy specimens from patients with long-segment Barrett's oesophagus. In contrast this pattern was not seen in any of the gastric cardia biopsies or gastric resection specimens. On the basis of these results the authors suggested that cytokeratin staining could be used to reliably distinguish Barrett's oesophagus from intestinal metaplasia of the stomach.

The present study, looking at supra-anastomotic biopsies from the oesophageal remnant in patients who had undergone sub-total oesophagectomy found the

classical CK7/20 staining pattern in 62% of cases and in 83% of a subgroup of patients with columnar metaplasia with specialised intestinal metaplasia. This prevalence is clearly less than that reported by Ormsby and the potential reasons for this warrant further exploration.

Following the Ormsby paper, several groups undertook studies evaluating cytokeratin staining of both normal and metaplastic tissues from the region of the oesophago-gastric junction with mixed results. There is therefore some suggestion that the original paper may overestimate the prevalence of this CK7/20 staining pattern in sporadic Barrett's oesophagus.

Several groups have reported similar findings to Ormsby with the classical CK7/20 staining pattern observed in over 90% of cases of Barrett's oesophagus (deMeester et al., 2002, Glickman et al., 2001, Jovanovic et al., 2002, Shearer et al., 2005). These studies include cases of both long and short-segment Barrett's oesophagus but all have included the presence of specialised intestinal metaplasia in the definition of Barrett's oesophagus. In contrast, El-Zimaity and Graham (2001), in a study of biopsy samples from long-segment Barrett's oesophagus found the classical pattern in only 45% of 29 patients. A further study by Mohammed and colleagues (2002) also found only moderate sensitivity for Barrett's oesophagus using the classical CK7/20 staining pattern. In this retrospective study involving 49 cases of both long and short-segment Barrett's oesophagus the classical pattern was seen in 65% of cases, when subdivided into patients with long and short segment Barrett's, the figures were 54% and 81% respectively. The findings in the present study of a 62% prevalence of the classical CK7/20 staining pattern overall and an 83% prevalence in patients with SIM are therefore similar to the literature reported prevalences.

Technical differences and inter-observer variability are a possible explanation for the discrepancies seen in the various studies. Slightly different immunohistochemical staining protocols have been used by investigators along with antibodies provided by different suppliers which may affect the appearance of the stained slides and the subsequent interpretation of the staining pattern. It is known that the fixative used can affect staining. Glickman et al. (2005)

showed poor inter-institutional agreement (71%) in the presence of the classical Barrett's CK7/20 pattern in patients with Barrett's oesophagus with specialised intestinal metaplasia. They identified that the major source of disagreement was in the interpretation of weak or variable CK7 staining of deep intestinalised mucosa of biopsies fixed in Hollande's solution but not those fixed in formalin. After new criteria were established, taking into account the effect of the different fixatives, inter-institutional agreement improved. The present study used formalin fixative throughout, as did the studies by El-Zimaity and Mohammed, but there may be other, unidentified technical differences affecting these studies which could explain the lower prevalence of the typical CK7/20 pattern. Specimens in the current study were assessed against a criteria described in a published paper, The paper included pictorial examples of the classical staining pattern but interpretation may have varied between the authors of the original paper and the present study, particularly in equivocal cases.

In the present study, all patients with neo-Barrett's in the oesophageal remnant were included provided they had given consent for biopsies to be used for this purpose. The advantage of this approach was that it maximised the amount of neo-Barrett's tissue to be studied but it may also contribute to the somewhat lower prevalence of the classical staining pattern observed when compared to other studies. Biopsies from the oesophageal remnant can be technically difficult to obtain. The areas of neo-Barrett's were, by definition, never long-segments and on occasion it was difficult to obtain reasonable sized samples whilst ensuring the accurate placement of the biopsy forceps. The principle concern being to ensure that there is no inadvertent sampling from the gastric side of the anastomosis which could lead to overdiagnosis of columnar metaplasia. Personal observations suggest that endoscopic biopsy in the cervical oesophagus can occasionally be poorly tolerated by patients due to the increased amount of retching when the endoscope is in this region. Despite the fact that pyloroplasty was routine for patients in this series, there was some evidence of delayed gastric emptying with food residue seen in the gastric conduit in many cases. On occasion food residue was present within the oesophageal remnant causing further problems with obtaining biopsy samples. These problems together meant that a number of samples in the present study were either small or demonstrated crush artefact making assessment of

immunohistochemical staining more challenging than for larger, better orientated specimens. It seems likely that in other retrospective studies, particularly those involving oesophagectomy specimens and long-segment Barrett's, the specimens to be assessed would have been of better quality. Indeed the seminal study by Ormsby reports that tissue blocks for the study were selected as they were felt to be the most representative.

In many cases in the present study the biopsy samples from individual patients contained more than one type of epithelium. In some cases there were differences between the various biopsy fragments and in other cases there was more than one type of epithelium within a single biopsy. Typical patterns seen, included biopsies with both squamous and columnar mucosa and biopsies with a small focus of intestinal metaplasia, within predominantly non-intestinalised columnar epithelium. This situation implies that neo-Barrett's in the oesophagus may be composed of a relatively heterogeneous epithelium, perhaps with different areas at different stages of development. Alternatively it may be that these differences simply reflect the problems with obtaining accurate biopsies given the technical difficulties outlined above. In either case the presence of heterogeneous samples undoubtedly led to problems assessing staining patterns. The scoring system employed suggested that the most representative areas of epithelium should be used to assess the staining pattern for the patients. Assessment of which area was most representative, however, introduces a further element of subjectivity to the scoring process. Patients with specialised intestinal metaplasia were assessed as a single group regardless of whether this was the predominant subtype of epithelium. It is possible that for patients with only a small focus of specialised intestinal metaplasia, this may have 'cut out' between the level of the slides cut for H&E assessment and those cut for immunohistochemistry assessment. In the two cases of type A Barrett's which did not stain with the classical Barrett's type cytokeratin pattern, the area of intestinal metaplasia identified on the H&E stained slide took the form of a small single focus. This suggests that intestinal metaplasia may not have been evident on subsequent slides which in turn could explain the lower prevalence of classical Barrett's pattern staining in this study compared to others.

The present study used the current British definition of Barrett's oesophagus, a segment of columnar metaplasia of any length visible endoscopically and confirmed or corroborated histologically. All of the major studies looking at cytokeratin expression in Barrett's oesophagus have used an American definition requiring the presence of specialised intestinal metaplasia (deMeester et al., 2002, El-Zimaity and Graham, 2001, Glickman et al., 2001, Jovanovic et al., 2002, Mohammed et al., 2002, Ormsby et al., 1999, Shearer et al., 2005). This difference could be an important explanation for the lower presence of a typical Barrett's staining pattern seen in the present study.

Despite the limitations of the samples in the present study outlined above, the prevalence of a typical Barrett's CK staining pattern was 83% in patients with Barrett's with intestinal metaplasia, higher than the prevalence for those without intestinal metaplasia. Squamous oesophageal epithelium does not normally express CK7 or CK20 which suggests that oesophageal cells must start to express these proteins at some point along the pathway of transformation to columnar metaplasia and Barrett's oesophagus. All 14 of the samples in the present study which did not stain with a typical Barrett's pattern exhibited some degree of CK7 and CK20 positivity. All but one demonstrated Barrett's like strong staining of the superficial epithelium for CK20 and the remaining sample demonstrated patchy staining. All samples demonstrated some degree of CK7 expression but this tended to be more patchy than is seen in the classical Barrett's pattern. Evidence from the present study outlined in the previous chapter suggests that Barrett's oesophagus with specialised intestinal metaplasia is a more advanced stage of the metaplasia-dysplasia-adenocarcinoma sequence than that without. It is therefore proposed that the samples in the present study represent relatively early stage Barrett's oesophagus which in some cases has not yet achieved the mature CK7/CK20 expression pattern but which is in the process of developing this.

Despite the above considerations, it is important to consider two other potential explanations for why the present study may have shown a lower prevalence of a classical Barrett's oesophagus cytokeratin staining pattern. These are namely that there could have been sampling errors, with biopsies taken from incorrect

anatomical conditions or that post-oesophagectomy neo-Barrett's is, in fact a different entity from sporadic Barrett's oesophagus.

As previously described, the site of the surgical anastomosis is clearly visible at endoscopy in the majority of cases making determination of the site of the gastro-oesophageal junction much easier than for other patients. Biopsies were taken by experienced endoscopists with a specialist interest in neo-Barrett's thereby minimising the risks of sampling errors. If errors in the site of biopsies were made however, one would expect tissue samples to resemble either normal oesophagus, above an area of neo-Barrett's or gastric body tissue from which the gastric conduit is fashioned.

Proximal sampling error, where biopsies are taken from above the segment of neo-Barrett's can be easily discounted by the presence of columnar epithelium. In addition the cytokeratin staining pattern described in squamous oesophagus is markedly different from that seen in the neo-Barrett's samples of the present study. Both Shearer and Ormsby report uniform absence of CK20 (Ormsby et al., 1999, Shearer et al., 2005). Shearer also reports uniform absence of CK7, although Ormsby reports a more varied picture with some cases showing a complete absence of CK7 and others exhibiting strong reactivity, particularly in areas adjacent to Barrett's epithelium.

Distal sampling error is more difficult to discount, but here too there are differences in the cytokeratin expression in the neo-Barrett's samples and those reported in gastric tissue. The comparison to be made is with the gastric body rather than the cardia which is removed in its entirety at subtotal oesophagectomy. In Ormsby's original study cytokeratin expression was studied in normal control tissues including gastric body samples. This showed surface and foveolar staining for CK20 but no staining for CK7. The study by Shearer et al included 20 gastric biopsy samples as controls and again surface and foveolar staining with CK20 and negative staining for CK7. In the present study all but one sample demonstrated CK7 staining in the superficial compartment and all samples demonstrated CK7 staining in the deep compartment. These results therefore strongly support the claim that the columnar epithelium of our neo-Barrett's samples, regardless of subtype, is

different from that of the adjacent normal tissues and does not simply represent a sampling error.

One further molecular marker was evaluated in the present study to assess the similarity of post-oesophagectomy neo-Barrett's to sporadic Barrett's oesophagus. Chromogranin A, a marker of neuroendocrine differentiation was present in all neo-Barrett's samples tested. The presence of neuroendocrine cells in sporadic Barrett's oesophagus has been described by several authors. Griffin and Sweeney (1987) detected neuroendocrine cells in 90% of biopsies and resections of Barrett's mucosa. Two later studies have assessed neuroendocrine differentiation in Barrett's using Chromogranin A immunohistochemistry (Hamilton et al., 2000, Koppert et al., 2004).

Hamilton demonstrated Chromogranin A expression in 21 of 34 cases (62%) of Barrett's oesophagus, again in a combination of biopsy and resection specimens whilst Koppert demonstrated Chromogranin A expression in 38 of 56 oesophagectomy specimens (68%) containing Barrett's mucosa. As in the present study both Hamilton and Koppert describe Chromogranin A positive cells as being infrequent and scattered with very occasional small clusters of cells.

There is clearly some discrepancy between the finding in the current study of universal Chromogranin A expression and the findings of Hamilton and Koppert which suggests that this is present in around two thirds of cases of sporadic Barrett's oesophagus. Unlike the present study, all samples in the previous studies were obtained from patients with adenocarcinoma of the oesophagus or oesophago-gastric junction. Adjacent areas of Barrett's could therefore be thought of as being at an advanced stage within an unstable oesophageal epithelium. In the neo-Barrett's patients of the current study there were no cases of dysplasia of any type and the mucosa of the oesophageal remnant was proven to have been squamous at a median of only 6.6 years previously.

There is some evidence from the studies of Hamilton and Koppert to suggest that Chromogranin A expression in Barrett's may be lost as the condition progresses. Hamilton describes 3 cases where Chromogranin A expression

was lost in areas of high grade dysplasia in patients with Chromogranin A positive areas of low grade dysplasia or non-dysplastic Barrett's. In Koppert's study it was observed that neuroendocrine cells were found more often in Barrett's with no dysplasia or low grade dysplasia compared to that with high grade dysplasia although this difference was not statistically significant. It may therefore be the case that the increased rate of Chromogranin A expression seen in the present study represents the fact that this is relatively early stage columnar metaplasia with no evidence of progression to dysplasia.

An alternative explanation may relate to the high levels of proton pump inhibitor (PPI) use in the present study as there is a reported association between PPIs and neuroendocrine proliferation. These drugs act by blocking the action of the proton pump of gastric parietal cell membranes. This pump directly secretes hydrogen ions into the gastric lumen in exchange for potassium ions and inhibition of this process therefore results in profound acid suppression. Gastrin, secreted by the gastric antral G cells is involved in the secretion of gastric acid and is part of the feedback mechanism for controlling acid production. Gastrin secretion is inhibited in the presence of high levels of gastric acid and profound acid suppression, such as that induced by PPIs suppresses this negative feedback process resulting in hypergastrinaemia (Laine et al., 2000). In addition to its secretory effects, gastrin has a trophic effect on the oxyntic mucosa in general and on neuroendocrine cells called enterochromaffin-like (ECL) cells in particular (Bakke et al., 2000). In rats, both surgically and proton pump induced acid suppression has been shown to result in hypergastrinaemia, ECL hyperplasia and formation of ECL carcinoid tumours (Mattsson et al., 1991, Havu, 1986). In humans the evidence is more contradictory, PPI induced hypergastrinaemia has been shown to be associated with ECL hyperplasia but there is no convincing evidence that there is a significant association with carcinoid tumours (Lamberts et al., 1993 , Brunner et al., 2012).

Of the 37 patients in the present study, 27 (73%) reported current or long-term proton pump inhibitor (PPI) use. In addition to the association between PPI use and ECL hyperplasia it has been shown that long term PPI use is associated with elevated serum Chomogranin A levels (Sanduleanu et al., 1999). It is

possible that the widespread presence of neuroendocrine cells as evidenced by chromogranin A staining in the present study is, at least in part, related to the high prevalence of PPI use in the study population.

Very few other studies have evaluated molecular marker expression in neo-Barrett's tissue and those that have involved much smaller numbers of patients. As in the present study however, the limited data which has been published suggests that neo-Barrett's is characterised by the expression of the same molecular markers as is seen in sporadic Barrett's oesophagus.

Lord and colleagues (2004) used immunohistochemical techniques to evaluate cytokeratin 7 and 20 expression. This study included 10 samples of cardiac type neo-Barrett's and 4 samples of neo-Barrett's with specialised intestinal metaplasia.(Lord et al., 2004) As in the present study, there was superficial staining for both of these cytokeratins with deep glandular staining for cytokeratin 7 also present. This pattern was seen in neo-Barrett's tissue of both types but staining was observed to be more intense in areas of intestinal metaplasia. Bax et al (2007) also studied cytokeratin 7 and 20 expression in 23 patients with neo-Barrett's and found CK7 and CK20 to be present in a Barrett's like pattern in all samples(Bax et al., 2007).

Barrett's epithelium is characterised by the presence of goblet cells and expression of intestinal markers including MUC2 (a secretory mucin), alkaline phosphatase and isomaltase. There is increasing evidence that neo-Barrett's epithelium is characterised by the presence of the same intestinal markers. The enterocytic enzyme isomaltase was identified in samples from 2 out of 4 patients with neo-Barrett's by Chaves and collegues (2002). More recently Bax (2007) described similar expression of the mucin gene product MUC2 in neo-Barrett's samples as is seen in Barrett's. The antibody DAS1 reacts with goblet cells. Immunhistochemical staining with this antibody is positive in Barrett's but not squamous oesophageal mucosa. Neo-Barrett's mucosa with intestinal metaplasia, stains strongly for DAS1, providing further evidence of phenotypic similarity to sporadic Barrett's (Lord et al., 2004). CDX2, a protein involved in intestinal differentiation, predominantly expressed in the small intestine and colon of adults is also known to be present in Barrett's tissue. In neo-Barretts

tissue CDX2 expression appears frequently in samples with intestinal metaplasia and infrequently in samples with gastric-type metaplasia perhaps providing further evidence that non intestinalised columnar epithelium is an early form of columnar metaplasia (Bax et al., 2007).

In summary the cytokeratin 7 and 20 staining pattern seen in the neo-Barrett's biopsy samples in the present study was similar to that reported in sporadic Barrett's oesophagus. A typical Barrett's like pattern was seen in 62% of cases, and whilst this prevalence is lower than that reported in some studies it is within the range of values reported in the literature for sporadic Barrett's oesophagus. In addition the pattern is different from that expected in the adjacent tissues. Chromogranin A expression is seen in neo-Barrett's tissue in a similar pattern to that described in sporadic Barrett's oesophagus although it is seen more frequently. These findings together suggest that neo-Barrett's oesophagus is molecularly similar to sporadic Barrett's oesophagus and support the hypothesis that post-oesophagectomy neo-Barrett's is an appropriate model for the development of Barrett's oesophagus.

Chapter 5. Expression of Trefoil Factors in neo-Barrett's epithelium

5.1 Introduction

Trefoil factors are a family of proteins which contain the three-loop trefoil domain. TFF1 and TFF3 are able to form dimers and an example of the structure is shown in figure 5.1. Three human trefoil factors have been identified. The predominant site of expression of all three is within the gastrointestinal tract however there are differences in the precise locations of expression.

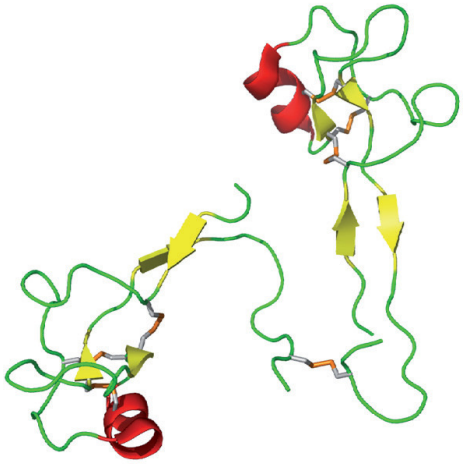


Figure 5.1: Structure of human TFF1 dimer. Note the three loops in each globular trefoil domain creating a three-leafed structure. The red region indicates the short region of α-helix that is present in the second loop of the trefoil domain (Kjellev, 2009) reproduced with kind permission of Springer Science)

The function of trefoil proteins remains far from certain. They are relatively resistant to heat, acid and enzymes and it has been proposed that they may interact with mucin molecules in order to stabilise the mucus gel which protects the epithelium of the gastrointestinal tract (Wong et al., 1999).

In addition to their proposed protective role, it has been suggested that trefoil proteins may have a role in the repair of damaged tissue. They have been identified in what has been termed the ulcer associated cell lineage (Hauser et al., 1993). This cell lineage forms a unique glandular structure at sites of chronic gastrointestinal ulceration including those seen in Crohn's disease and peptic ulcers (Wright et al., 1990). Trefoil proteins appear to be able to

stimulate the migration of cells from the healthy epithelium at the edge of a wound (Playford et al., 1995, Dignass et al., 1994). These cells are stimulated to move to cover the damaged area allowing rapid repair and protection from further damage.

In addition to their role in mucosal protection and repair there is evidence to suggest that trefoil proteins have a role in carcinogenesis. Mice lacking the gene for TFF1 expression are prone to gastric adenomas and adenocarcinomas and there is increasing evidence that abnormal TFF expression is a common feature of many types of tumour (Lefebvre et al., 1996) (Kjellev, 2009) (Regalo et al., 2005).

The finding of an association between the trefoil factors and both ulceration and cancer within the gastrointestinal tract has led to interest in the study of trefoil proteins in Barrett's oesophagus. Barrett's is believed to develop in response to chronic gastroduodenal reflux and the resultant inflammation. It has been suggested that the columnar metaplasia seen in Barrett's is an adaptive response to this reflux related injury with the metaplastic columnar epithelium being more resistant to acid than the native oesophageal squamous mucosa. This metaplastic epithelium may be able to secrete protective mucins as happens elsewhere in the gastrointestinal tract and trefoil proteins could clearly have a role in this. In addition, secretion of trefoil proteins in the distal oesophagus could potentially aid mucosal repair following reflux, where damage has occurred. A small number of studies have demonstrated expression of trefoil factors in Barrett's oesophagus providing some evidence for these theories (Hanby et al., 1994, Warson et al., 2002, Labouvie et al., 1999, Fox et al., 2005, Van de Bovenkamp et al., 2003).

The stage of the metaplastic process at which trefoil factors start to become expressed is unclear as is the expression pattern for the different subtypes of Barrett's oesophagus. Expression of trefoil factors has never been specifically studied in the proximal oesophagus or in a post oesophagectomy neo-Barrett's population.

The aim of the present study was to assess the expression of the three human trefoil factors in neo-Barretts tissue. As outlined in previous chapters the oesophageal remnant following surgery potentially provides a unique opportunity to study the early stages of Barrett's oesophagus. By evaluating the expression of trefoil proteins in this environment it was hoped to gain some insight into their role in the development of Barrett's oesophagus and also to compare their expression here with what has been observed in sporadic Barrett's oesophagus.

5.2 Methods

Patients and inclusion criteria for the study were as outlined in the previous chapter. All had confirmed neo-Barrett's with evidence of a squamous lined oesophageal remnant at the time of surgery.

Thirty five patients were endoscoped prospectively by, or in the presence of the author. The remaining two patients were identified retrospectively and had clear documentation and picture evidence of columnar epithelium above the anastomosis from which biopsies had been taken. Biopsy technique and initial processing was as outlined in the previous chapter.

5.2.1 Trefoil factor immunohistochemistry

Monoclonal antibodies raised in mice (a gift from F. May) were applied to the biopsy sections according to the protocol described in appendix G. An avidin-biotin-peroxidase method was used with 3,3'-diaminobenzidine as a chromogen to view positive binding.

5.2.2 Assessment and scoring of samples

Histological classification was determined by an expert gastrointestinal pathologist. Where more than one type of epithelium was present, classification was based on the predominant type with the exception of samples containing intestinal metaplasia. Samples containing any areas of intestinal metaplasia were classified as type A. In these cases immunohistochemical scoring was based only on representative areas of epithelium.

Staining for all three trefoil factors was assessed for both the superficial surface epithelium and the deeper glandular structures, only epithelial cells were considered. Immunohistochemical scoring was carried out by the author using a semi-quantitive scoring method as described in table 5.1.

Score	Description
0	Staining absent
1	Staining in 0 – 25% of cells
2	Staining in 25 – 50% of cells
3	Staining in 50 – 75% of cells
4	Staining in 75 – 100% of cells

.
Table 5.1: Semi-quantitative scoring system used to assess trefoil staining

5.2.3 Statistical analysis

Comparisons of proportions were undertaken using the Fisher's exact test or Mann-Whitney U tests as appropriate.

5.2.4 Ethical approval

Ethical approval was sought and granted by the County Durham and Tees Valley 2 REC as outlined previously.

5.3 Results

5.3.1 Study population

The study population comprised a total of 37 patients, 31 males and 6 females. Median time following surgery was 6.6 years (range 1.0 – 13.2 years).

5.3.2 TFF1

Normal stratified squamous epithelium was usually seen in the sections of the biopsies, often continuous with the columnar epithelium. TFF1 was not expressed by any of the normal squamous epithelium.

TFF1 was extensively expressed by the columnar epithelium, predominantly in the superficial compartment. All samples demonstrated extensive 4+ staining of the superficial epithelium (figure 5.2). Within the surface epithelium, two distinct patterns of staining were seen. In one there was equal staining throughout the cytoplasm and in the other the staining had a clear apical predominance (figure 5.3). Twenty two specimens (60%) demonstrated the predominantly apical staining phenotype.

TFF1 was expressed by the deeper glandular tissues in the majority of cases but the extent of this staining was much less than in the superficial compartment (figure 5.2).

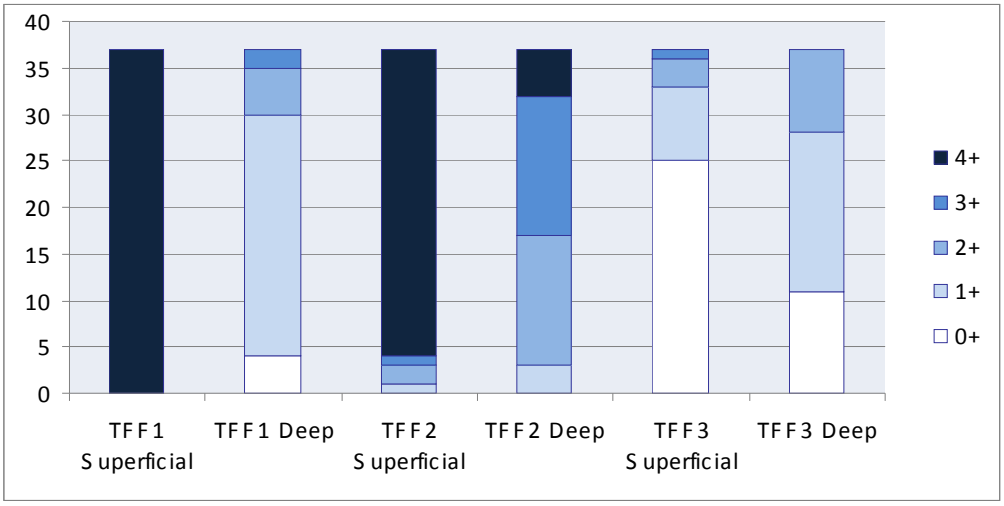


Figure 5.2: Extent of Trefoil factor staining within superficial and deeper glandular tissues of columnar epithelium

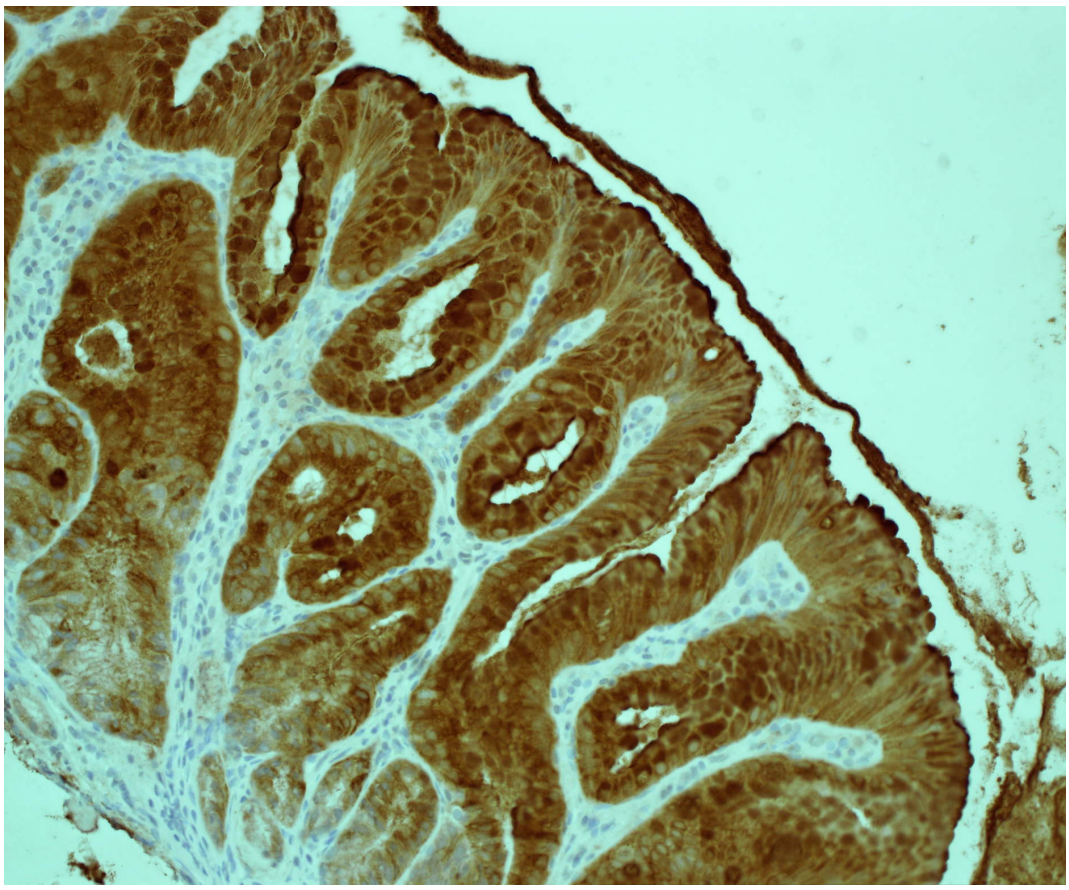


Figure 5.3a: TFF1 Staining where there is equal staining throughout the cytoplasm

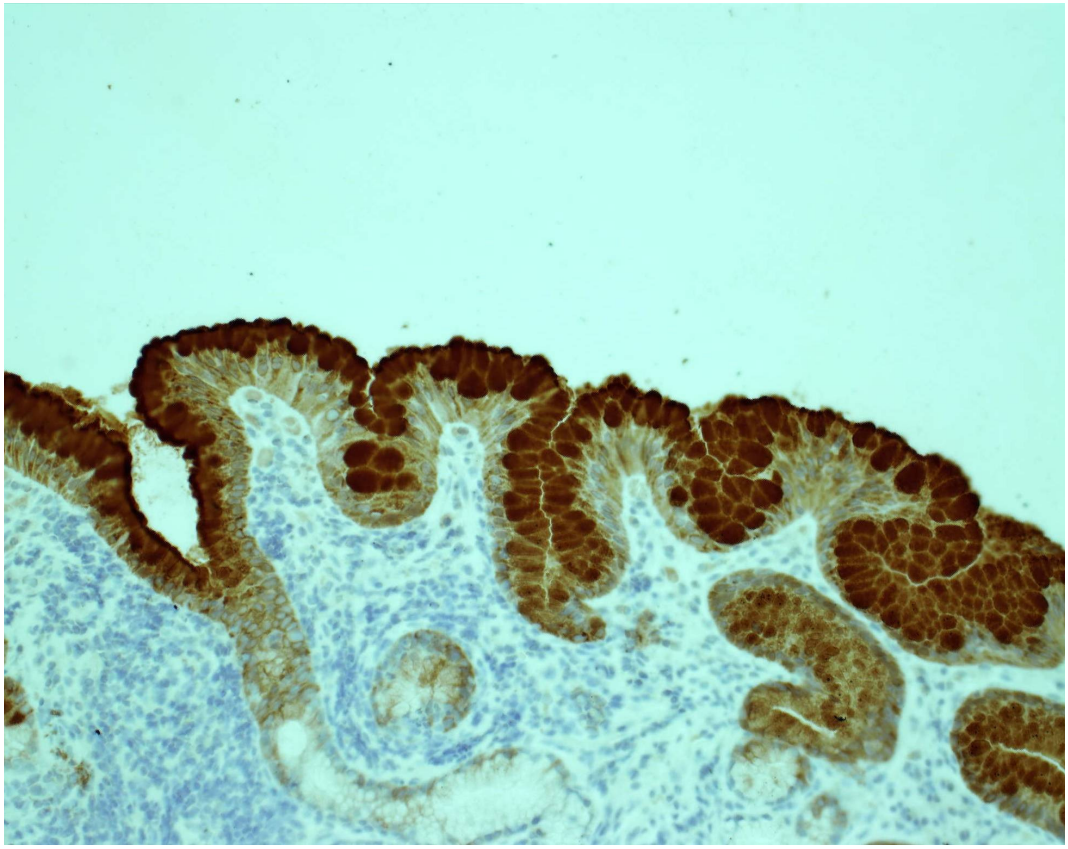


Figure 5.3b: TFF1 staining where there is an apical predominant pattern

There was a trend towards more extensive expression of TFF1 in specimens with intestinal metaplasia compared to those without (figure 5.4) but this just failed to reach statistical significance (p=0.058).

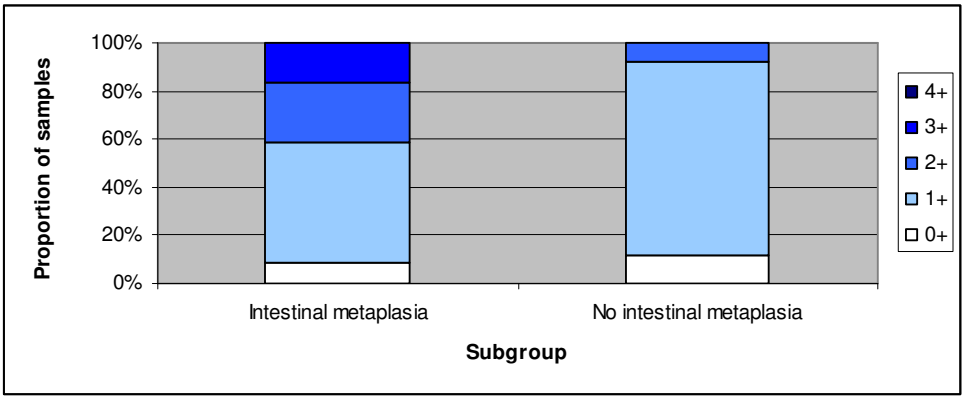


Figure 5.4: Extent of TFF1 in deeper glandular tissues according to subtype of columnar metaplasia

5.3.3 TFF2

There was no expression of TFF2 in the areas of normal stratified squamous epithelium present within the specimens.

TFF2 was the most widely expressed of the three trefoil factors with the majority of specimens demonstrating strong (4+) superficial staining in addition to marked staining of the deeper glandular structures (figure 5.2). There was no difference in either the superficial or deep staining for TFF2 according to the presence of intestinal metaplasia (p=1.000 and p=1.000 respectively (figures 5.5 and 5.6).

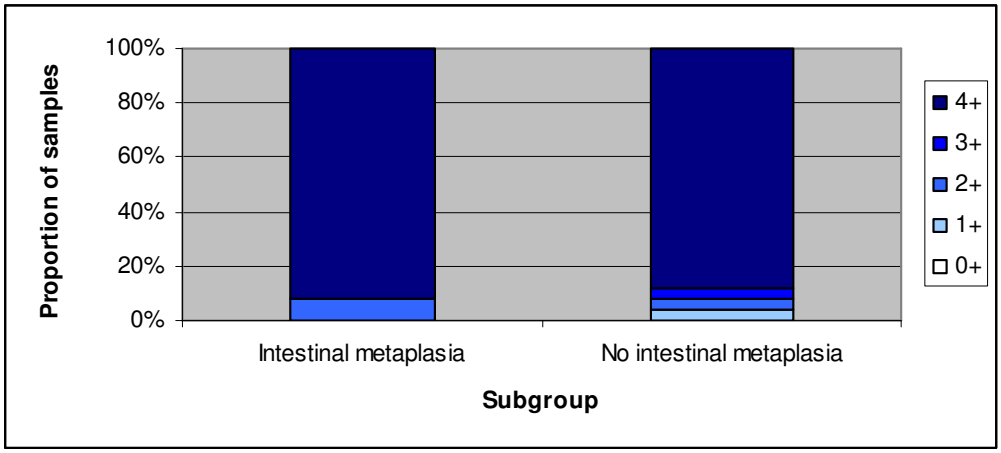


Figure 5.5: Extent of TFF2 in superficial epithelium according to subtype of columnar metaplasia

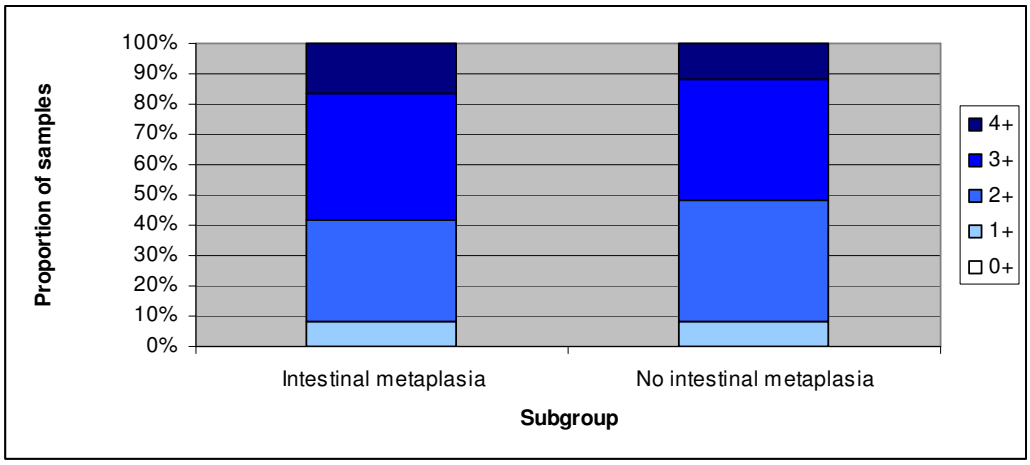


Figure 5.6: Extent of TFF2 in deeper glandular tissue according to subtype of columnar metaplasia

5.3.4 TFF3

There was no expression of TFF3 in areas of normal stratified squamous epithelium present within the specimens.

TFF3 was the least widely expressed of the three trefoil factors with only 1 specimen demonstrating more than 50% staining in the superficial epithelium and no samples demonstrating more than 50% staining in the deeper glandular tissues (figure 5.2). When present, the staining was diffuse and cytoplasmic with no cases of an apical predominant pattern.

There was no significant difference in the staining intensity for TFF3 in either the superficial or deep compartments for columnar metaplasia with intestinal metaplasia compared to columnar metaplasia without intestinal metaplasia ($p=0.074$ and $p=0.081$ respectively) (figures 5.7 and 5.8). Only one specimen of type A and one specimen of type C columnar epithelium demonstrated a complete absence of staining for TFF3. There were 6 specimens of type C columnar epithelium which demonstrated no TFF3 staining however this difference did not reach statistical significance.

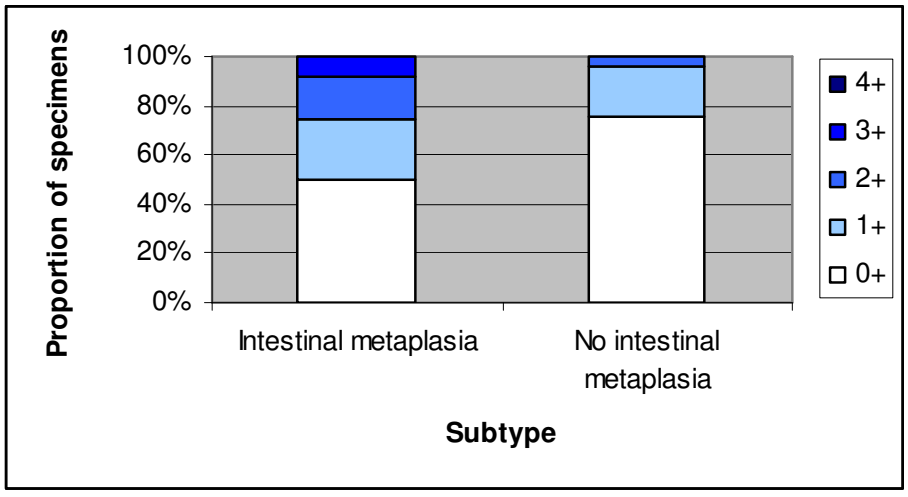


Figure 5.7: Extent of TFF3 in superficial epithelium according to subtype of columnar metaplasia

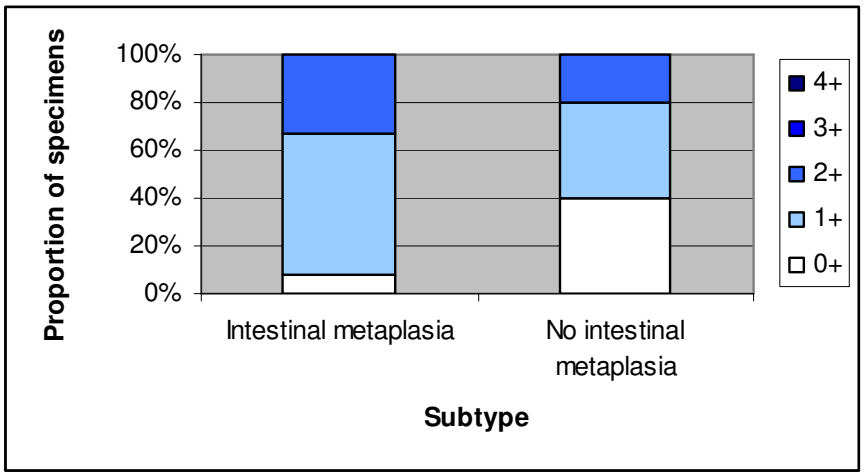


Figure 5.8: Extent of TFF3 staining in the deeper glandular tissue according to subtype of columnar metaplasia

5.3.5 Time from surgery and TFF expression

There was a significant association between the time that had elapsed since surgery and the presence of TFF3. Patients whose samples expressed TFF3 were endoscoped at a median of 8.1 years following surgery compared to a median of 3.4 years for those with no TFF3 expression (p=0.004). This association was not seen for the other 2 trefoil factors (figure 5.9). In the case of TFF2, all samples demonstrated some staining regardless of time from surgery. There was greatest variability in the expression of TFF1 and TFF2 in the deeper glandular tissue and it was therefore decided to assess whether there was any correlation between the extent of expression of these trefoil factors in the deeper tissues and the time from surgery. There was however no significant correlation (p=0.292 and p=0.084 respectively) (figure 5.10). For the other staining scores the variability was too limited for correlation analysis to be relevant.

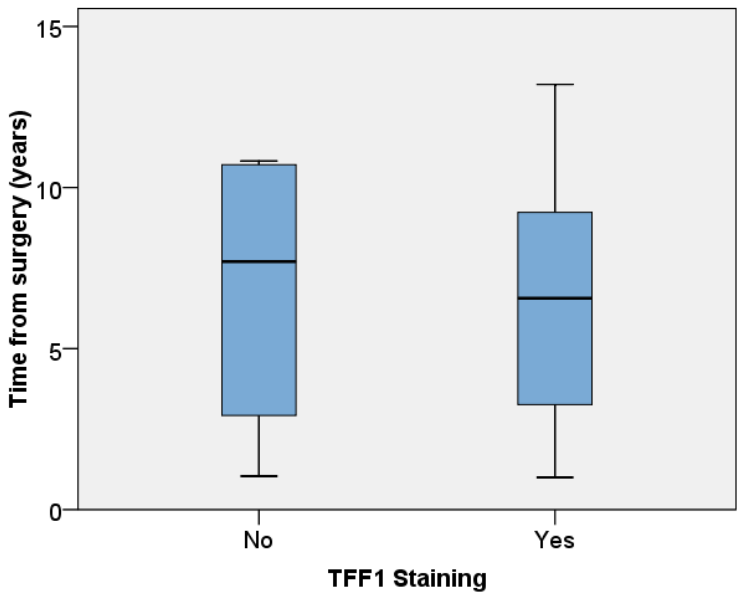


Figure 5.9a: Association between time from surgery and the presence of TFF1 staining p=0.696 (Mann-Whitney)

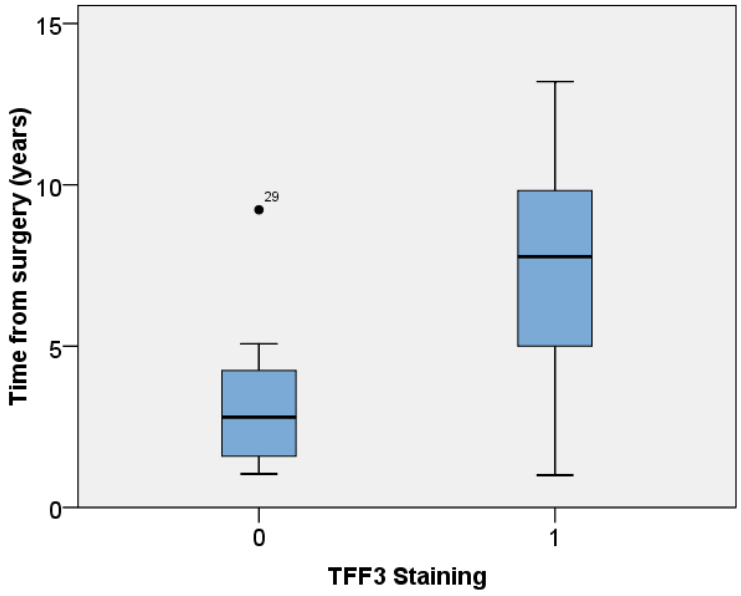


Figure 5.9b: Association between time from surgery and the presence of TFF3 staining p=0.004 (Mann-Whitney).

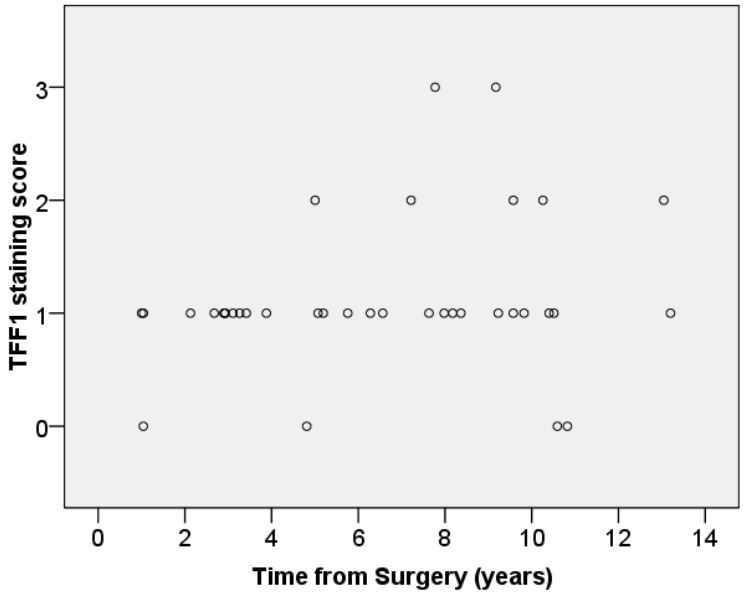


Figure 5.10a: Association between time from surgery and the staining score for TFF1 in deeper glandular structures. There was no significant correlation (p=0.292)

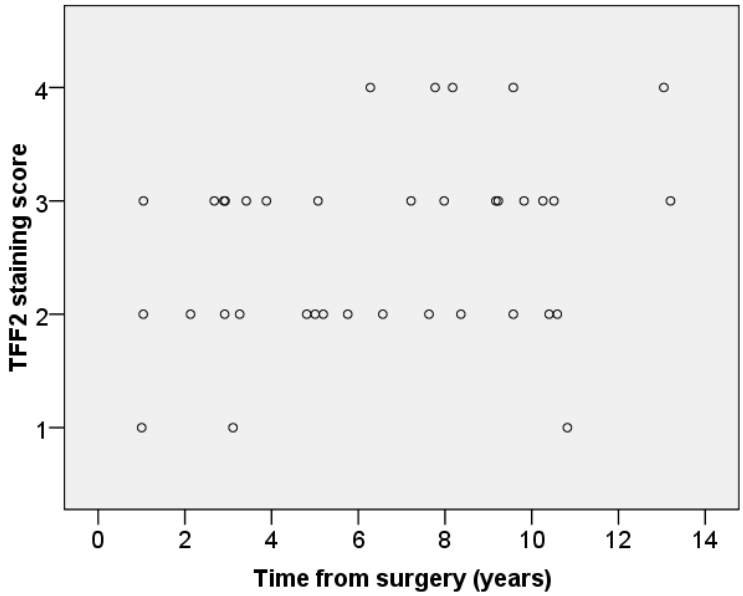


Figure 5.10b: Association between time from surgery and the staining score for TFF2 in deeper glandular structures. There was no significant correlation (p=0.084)

5.3.6 Pattern of staining

In a proportion of specimens the trefoil factor staining took a distinctive form with circular areas of strong staining within goblet cells (figure 5.11). Where this

appearance was noted it was present for all 3 trefoil factors in the majority of cases (table 5.2).

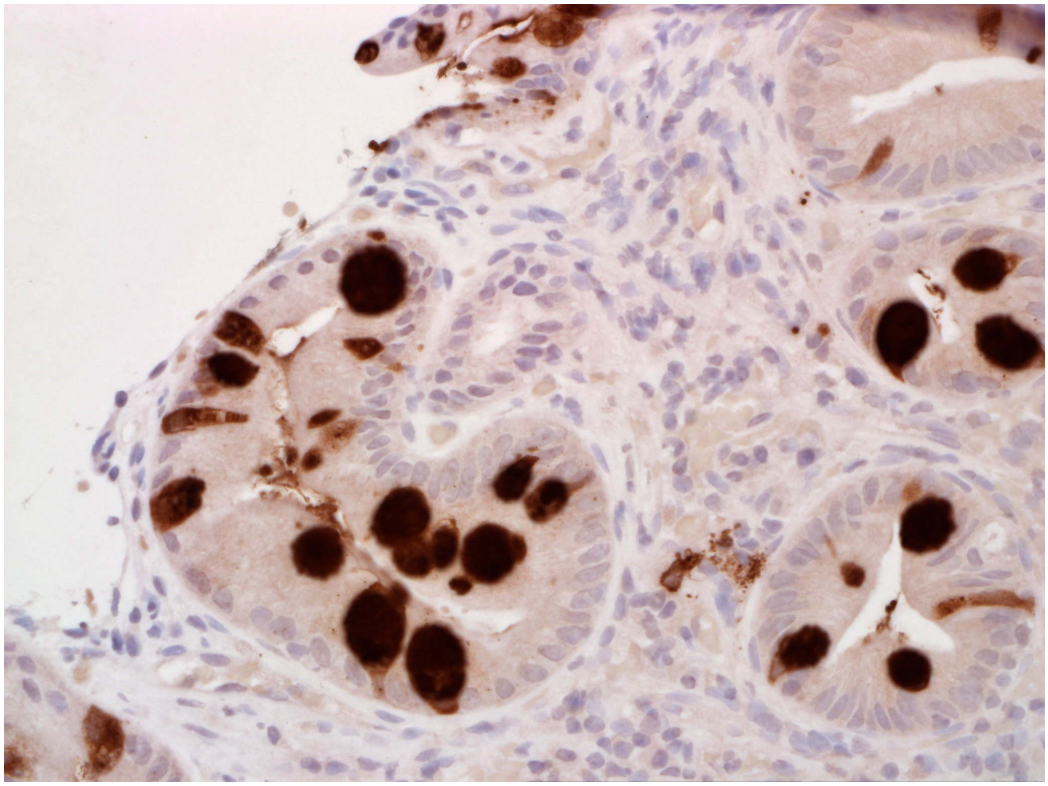


Figure 5.11a: High powered view of strong circular areas of staining for TFF3 in goblet cells in a specimen with specialised intestinal metaplasia

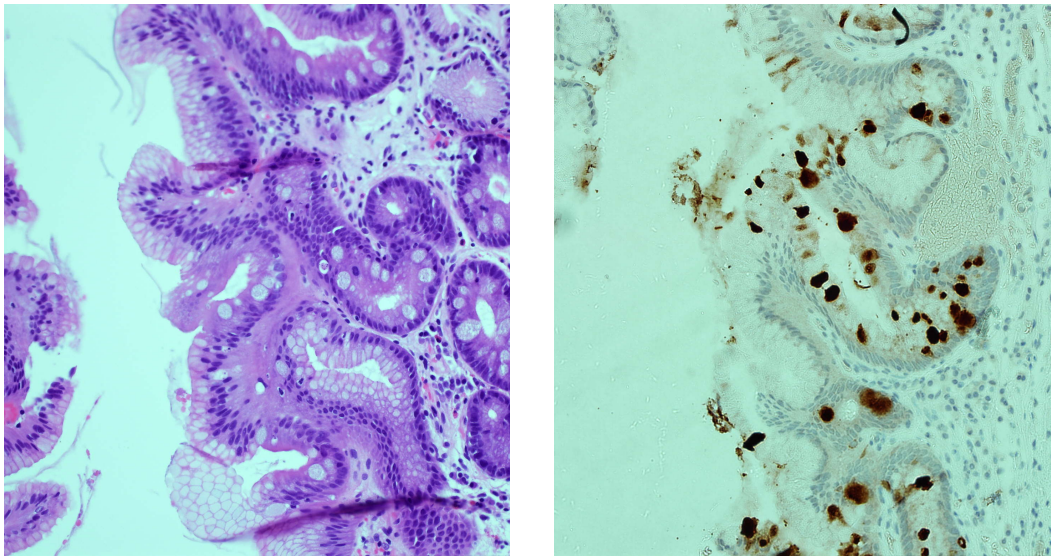


Figure 5.11b: Low powered view H&E stained neo-Barrett's with corresponding slide showing TFF3 staining in goblet cells

Patient	Distinct goblet cell staining for TFF1	Distinct goblet cell staining for TFF2	Distinct goblet cell staining for TFF3
1	No	No	No
2	Yes	No	Yes
3	Yes	Yes	Yes
4	Yes	Yes	Yes
5	Yes	Yes	Yes
6	Yes	Yes	Yes
7	No	No	No
8	No	No	No
9	No	Yes	Yes
10	Yes	Yes	Yes
11	Yes	Yes	Yes
12	No	No	No

Table 5.2: Distinctive staining of goblet cells for trefoil factors in patients with neo-Barrett's with specialised intestinal metaplasia

5.4 Discussion

Trefoil factors are a family of proteins which bear the three-loop trefoil domain. There are three human trefoil factors, TFF1, TFF2 and TFF3. The first trefoil protein to be identified was TFF1. Initially named pS2, it was identified in a breast carcinoma cell line and was first described in the early 1980s (Masiakowski et al., 1982). TFF2 was identified around the same time having been extracted from porcine pancreas (Jorgensen et al., 1982). It was several years later, in 1989 that the similarity between these proteins was appreciated and the trefoil domain identified (Thim, 1989). TFF3 was the last of the trefoil factors to be discovered, initially described in the rat it was first termed intestinal trefoil factor (Suemori et al., 1991). The genes for the three human trefoil factors are clustered together on chromosome 21q22.3. The predominant physiological site of expression of all three is the gastrointestinal tract where they are expressed in a site specific pattern. Physiological expression has however been described in several sites outside the gastrointestinal tract including salivary gland, respiratory tissues and breast (Madsen et al., 2007).

The physiological function of trefoil proteins is relatively poorly understood but it is believed that they have an important role in the protection and repair of mucosal surfaces. Studies have shown that trefoil proteins are located within the mucin granules in mucous secreting epithelial cells and it has been suggested that trefoil proteins interact with the mucin molecules to stabilise the mucous gel present on gastrointestinal mucosal surfaces (Ahnen et al., 1994) (Wong et al., 1999). Longman and colleagues (Longman et al., 2000) used immunofluorescent co-labelling to demonstrate that each trefoil protein co-localises with a specific mucin and that the expression of these varies according to location within the gastrointestinal tract. TFF1 is highly expressed in the surface epithelial cells of the stomach and has also been reported to be expressed in the upper ducts and surface cells of Brunner's glands in the duodenum(Hanby et al., 1993b) (Hanby et al., 1993a). TFF2 is also mainly expressed in the stomach. In normal gastric mucosa high levels of expression have been observed in the mucous glands of the body and antrum as well as in the Brunners glands of the duodenum. In contrast TFF3 is predominantly expressed in the intestine by the goblet cells (Podalsky et al., 1993). It is also

more widely expressed than either TFF1 or TFF2 in non-gastrointestinal tissues including breast, uterus, hypothalamus and pituitary gland (Regalo et al., 2005).

There has been significant interest in the role of trefoil proteins in mucosal repair. All three trefoil factors have been found to be expressed in what is termed the ulcer associated cell lineage (UACL). This novel cell lineage was first described by Wright and colleagues (1990) and is found in proximity to ulcers in Crohn's disease and peptic ulceration. The acinar structures of the UACL are thought to arise as a direct result of the ulceration. They are able to secrete a number of molecules which are thought to be important in epithelial repair including trefoil proteins and epidermal growth factor. Trefoil proteins appear to be motogens, able to promote cell migration without affecting cell division. *In vitro* studies using wounded monolayers of cells have shown that addition of trefoil proteins results in a marked increase in the rated of epithelial migration into the wound (Dignass et al., 1994, Playford et al., 1995). This evidence suggests that trefoil proteins have an effect on ulcer healing by stimulating the migration of surviving cells from the edge of the damaged region to cover the damaged region itself.

In vivo data on the role of trefoil proteins comes from studies of 'knockout' mice. TFF1 knockout mice exhibit decreased mucous secretion and develop gastric antral adenomas and multifocal intramucosal adenocarcinomas (Lefebvre et al., 1996). TFF3 knockout mice in contrast do not appear to have an obvious alteration in phenotype but have an impaired response to intestinal damage. Administration of dextran sulphate sodium, an agent which causes mild epithelial injury in wild type mice, resulted in much more severe damage in TFF3 knockouts where poor mucosal healing and death secondary to colitis were observed (Mashimo et al., 1996). More recent work involving TFF2 knockout mice has suggested that TFF2 is able to down-regulate gastric acid secretion and that absence of TFF2 results in a susceptibility to non steroidal anti-inflammatory drug (NSAID) induced gastric ulceration (Farrell et al., 2002). The prevalence of gastric adenocarcinomas in TFF1 knockout mice has resulted in the hypothesis that the gene encoding for TFF1 may have a role as a tumour suppressor gene. Immunohistochemical studies of gastric tissue have suggested that whilst TFF1 is almost universally present in samples of normal

gastric mucosa, expression is lost in up 50% of gastric adenocarcinomas (Henry et al., 1991, Muller and Borchard, 1993, Machado et al., 1996).

Barrett's oesophagus is believed to develop in response to chronic mucosal injury in the presence of duodeno-gastro-oesophageal reflux. Given this proposed mechanism of epithelial damage, inflammation and attempted repair there is interest in the potential role of trefoil factors in the development of columnar metaplasia. Similarly the potential role of genes encoding for trefoil proteins as tumour suppressor genes raises the question of whether these molecules might be important in the malignant progression of Barrett's and the development of oesophageal adenocarcinoma. Trefoil proteins have also been proposed as potential biomarkers to assist in the diagnosis of Barrett's oesophagus (Lau-Sirieix et al., 2009).

In the present study, many specimens included areas of squamous epithelium alongside the metaplastic columnar epithelium. There was no evidence of trefoil protein expression in any of the squamous epithelium. This finding is similar to that of other studies which have assessed trefoil expression in squamous oesophageal mucosa (Hanby et al., 1994, Warson et al., 2002, Kouznetsova et al., 2007). A single study has found evidence of TFF3 expression within the squamous lined oesophagus. This study identified TFF3 messenger RNA (mRNA) in surgical resection specimens (Kouznetsova et al., 2007). Laser capture dissection and immunofluorescence localisation suggested that the TFF3 was expressed in the submucosal glands and not in the superficial stratified squamous epithelium likely to be sampled by plain biopsy, potentially explaining the absence of TFF3 noted elsewhere. In the present study the fragments of squamous mucosa came from areas directly adjacent to the columnar epithelium and must therefore have been exposed to the same luminal conditions. The absence of trefoil expression in this situation suggests that TFF1 and TFF2 cannot be secreted by squamous oesophageal mucosa in response to injury and the only way the protective and reparative effects of these trefoil proteins can be utilised in the oesophagus is via the adaptive metaplastic process seen in Barrett's oesophagus.

TFF1 expression is well described in Barrett's oesophagus, including the cardiac and fundic types (Hanby et al., 1994, Warson et al., 2002). TFF1 is predominantly found in the superficial epithelial compartment but some staining of deeper glands is described (Hanby et al., 1994). In the present study examining neo-Barrett's, extensive TFF1 staining (>75% of cells) was found in all samples of columnar metaplasia. The majority of the deeper glands also expressed TFF1 but staining was less extensive than in the superficial compartment. These findings are in agreement with what has been reported in cases of sporadic Barrett's oesophagus and also closely resembles the expression found in normal gastric tissue (Hanby et al., 1994, Warson et al., 2002, Labouvie et al., 1999, Fox et al., 2005) (Longman et al., 2000, Rio et al., 1988).

The TFF1 gene is recognised as a tumour suppressor gene in gastric adenocarcinoma however its role in oesophageal adenocarcinoma is less clearly defined. One study (Fox et al., 2005) has assessed TFF1 expression in adenocarcinomas of the oesophagus (n=31) and the gastro-oesophageal junction (n=72). This study found negative to weak TFF1 expression in tumours which contrasted with the abundant expression seen in Barrett's oesophagus tissue. The authors suggest, on this basis, that loss of TFF1 expression may be important in the malignant transformation of Barrett's oesophagus. In the present study all samples demonstrated abundant expression of TFF1, which may therefore indicate that these patients are at low risk of developing Barrett's carcinomas in the near future. The role of TFF1 in the malignant progression of Barrett's however remains to be fully elucidated and temporal studies and validation are required before this can be considered as a clinical biomarker.

TFF2 was the most extensively expressed trefoil factor in this study with extensive expression in the superficial compartment and much greater expression than TFF1 in the deeper glands (figure 4.2). In sporadic Barrett's oesophagus, studies of TFF2 expression have produced inconsistent results. Warson et al (2002) undertook immunohistochemical evaluation of Barrett's oesophagus biopsies and demonstrated similar results to the present study with TFF2 expression present throughout the surface epithelium and the deeper glandular structures. Hanby and colleagues (1994) demonstrated expression of

TFF2 mRNA in both the superficial and deeper tissues but found TFF2 protein to be predominantly located within the deeper compartment. They suggest that the discrepancy may to due to low levels of protein being made in the superficial epithelium under normal circumstances or it's rapid secretion although if this were the case it is unclear why this problem was not experienced in either the present study or in that by Warson. Labouvie et al. (Labouvie et al., 1999) failed to demonstrate any TFF2 expression in 21 biopsy samples of Barrett's metaplasia despite requiring staining of only 5% of cells to define positivity. In the context of disagreement with other studies and a lack of data regarding the positive controls used in this series concern remains that the reported absence of TFF2 is simply an anomalous result.

TFF3 was the least widely expressed of the trefoil factors in the present study with no cases of extensive (>75% of cells) staining. Two distinct patterns of staining were observed, one being a weak cytoplasmic pattern and the other being strongly positive staining associated with goblet cells in areas of intestinal metaplasia. Goblet cell associated TFF3 expression is described in the intestine and this type of strong round staining associated with goblet cells has been described in Barrett's oesophagus by others (Podalsky et al., 1993) (Lau-Sirieix et al., 2009). In contrast, the study by Warson et al. (Warson et al., 2002) found TFF3 to be absent from the goblet cells of intestinal metaplasia in Barrett's.(Warson et al., 2002) The authors also found no association between TFF3 expression and the presence of intestinal metaplasia in individual patients.

This paper also details the expression of mucins in Barrett's oesophagus. Mucins are glycosylated proteins which are produced and secreted in large amounts by epithelial cells in the gastro-intestinal tract (Kim and Ho, 2010). Up to 20 different types of mucin are recognised, each encoded for by a gene given the prefix MUC and numbered according to the order of discovery. Mucins can be broadly classified into two groups, secretory and membrane associated and they are produced in a tissue specific fashion. The structure of mucin molecules, with cysteine-rich domains allows them to form bonds with each other and with other molecules to form a highly viscous gel. In the gastrointestinal tract, mucins are the major component of the protective mucus-

gel layer which provides a defence against damage from ingested food and microbes and the acid and enzymes secreted as part of the digestive process. This gel layer also helps to lubricate the gut, assisting with transit of its contents. Abnormalities in mucin secretion are observed in several disease states including intestinal infections, inflammatory bowel disease and mucinous adenocarcinomas (Kim et al., 2005).

Warson and colleagues (2002) studied the expression of 4 major secretory mucins in Barrett's oesophagus including MUC2. MUC2 is considered to be a marker of intestinal differentiation associated with the presence of goblet cells. It is found in the normal intestine and has been found in intestinal metaplasia of the stomach (Chang et al., 1994, Reis et al., 1999). Despite the authors finding no significant association between TFF3 expression and intestinal metaplasia in individual patients they did show a correlation between the extent of MUC2 and TFF3 expression as determined in individual patients. This potentially provides circumspect evidence of a link between intestinal metaplasia and TFF3 expression in these samples.

A model for the secretory phenotype changes associated with the development of Barrett's metaplasia in the distal oesophagus has been proposed (Van de Bovenkamp et al., 2003). In this model the first step involves a change from the normal stratified squamous epithelium to a single layer of columnar epithelium forming glandular structures. At this stage virtually all cells produce secretory mucins and associated trefoil proteins suggesting that this metaplastic mucosa has an increased capacity for protection and repair in the face of ongoing reflux injury. In the next phase intestinal metaplasia with goblet cells develops, MUC2 expression increases dramatically and TFF3 expression increases in conjunction with this.

The present study has confirmed that a similar process is possible in the tissue of the proximal oesophagus following sub-total oesophagectomy. All types of columnar epithelium present in this area express trefoil proteins, whereas these proteins were never seen in residual areas of squamous epithelium. TFF3 expression was more common in neo-Barrett's of intestinal and cardiac types compared to that of gastric type. This difference failed to reach statistical

significance (p=0.129) but the numbers of each subtype were relatively small. There was however a statistically significant difference in the average time from surgery of patients demonstrating TFF3 expression compared to others. Median time from surgery was 8.1 years for patients expressing TFF3 compared to 3.4 years for patients with no TFF3 expression. This, along with the finding that the presence of intestinal metaplasia was associated with increasing time from surgery provides evidence to support the model outlined above. Ideally prospective studies collecting biopsy samples from the same patients over a prolonged period of time would be required to confirm this process however, on a practical level it is difficult to justify repeated endoscopies in this population without a good clinical indication.

TFF3 is present in the human stomach in very limited amounts only in the absence of intestinal metaplasia (Hauser et al., 1993). Peitz and colleagues (2004a) assessed TFF3 mRNA expression in the gastric cardia and compared this to the gastric body and the oesophageal columnar epithelium in patients with Barrett's with intestinal metaplasia. They found that TFF3 expression was significantly more frequent at the cardia (n = 15/24) than in the body of the stomach(n = 2/26). In addition, TFF3 at the cardia was more common in patients with gastro-oesophageal reflux disease compared to those without, although this difference was not significant. Patients with evidence of intestinal metaplasia at the cardia were excluded so, as in the present study, it appears that TFF3 expression can occur in the absence of intestinal metaplasia and goblet cells. Biopsies from the cardia included both cardiac-type and fundus-type mucosa. The authors found TFF3 expression less commonly in fundus-type mucosa than in cardiac-type but TFF3 was present in all cases of oesophageal intestinal metaplasia. Combining these results with those of the present study and others it is clear that TFF3 expression can occur in cardiac-type mucosa of both gastric and proximal and distal oesophageal origin. This supports the concept of cardiac mucosa being metaplastic, occurring in response to reflux and being a precursor to intestinal metaplasia. In the oesophagus the earliest metaplastic step may be conversion to a gastric fundic-type mucosa with only rare expression of TFF3.

One group has proposed TFF3 as a potential biomarker to be used in screening for Barrett's oesophagus (Lau-Sirieix et al., 2009). This group used microarray datasets to identify markers which were present in Barrett's oesophagus but absent in normal oesophagus and gastric mucosa. Validation was then performed using immunohistochemistry on biopsy samples. As predicted, TFF3 protein was expressed extensively at the luminal surface of Barrett's oesophagus and was absent in normal oesophagus and gastric mucosa. Of interest the gastric samples in this study were taken from the cardia, an area where Peitz (2004a) suggests that TFF3 expression is not uncommon in the presence of reflux. Despite this TFF3 expression in cells captured using an oesophageal sponge capsule technique does appear to have excellent specificity for Barrett's oesophagus and further studies of this technique are underway.

In summary the current study has demonstrated expression of trefoil factors in neo-Barrett's epithelium in a pattern similar to that seen in sporadic Barrett's oesophagus. The finding that TFF3 expression was associated with time from surgery suggests that evidence of this trefoil may indicate a more advanced stage of Barrett's.

Chapter 6. Genetic mutations in Barrett's oesophagus, associated oesophageal adenocarcinoma and subsequent neo-Barrett's oesophagus

6.1 Introduction

The normal adult oesophagus is lined by a stratified squamous epithelium which undergoes continuous renewal. As with other rapidly proliferating tissues the epithelium is maintained by a limited number of stem cells (Seery and Watt, 2000, Nicholson et al., 2012). These stem cells are able to divide to self-renew and to produce transit cells which differentiate to produce cells characteristic of the particular epithelium (Hall and Watt, 1989). The location of both normal oesophageal stem cells and Barrett's oesophageal stem cells remains unclear. Squamous stem cells are thought to be located in the interpapillary basal layer and possibly also in the glandular neck region of oesophageal submucosal gland ducts (Seery, 2002). There are several theories as to the origin and location of Barrett's stem cells but the evidence for these is even less conclusive.

There are five main theories as to the origins of Barrett's oesophagus:

1. Gastric cardia migration with subsequent intestinal metaplasia
2. A metaplasia of the oesophageal squamous epithelium
3. Circulating bone marrow stem cells which are multipotent and able to colonise a damaged oesophagus
4. A residual population of embryonic cells that persist at the gastro-oesophageal junction
5. Stem cells in oesophageal submucosal gland ducts

A summary of each theory is outlined below:

1. Gastric cardia migration with subsequent intestinal metaplasia

This was one of the earliest theories as to the origins of Barrett's oesophagus. In the canine experiments of Bremner et al. (1970) a columnar epithelium, similar to human cardiac epithelium, was observed to replace surgically excised oesophageal squamous epithelium in the presence of gastro-oesophageal reflux. Cardiac epithelium was thought to be more resilient and capable of migrating proximally to repair the denuded area of oesophagus (Bremner et al., 1970). This theory does not explain the presence of goblet cells in Barrett's oesophagus as these are not present in cardiac epithelium. The absence of

goblet cells in the normal gastric cardia does not however rule out this theory as intestinal metaplasia can occur in the stomach and the development of Barrett's via this mechanism could be explained by a two stage process of proximal migration with subsequent intestinalisation (Eda et al., 2003). Against this theory is the fact that cardiac type metaplasia has been observed above an intact ring of squamous epithelium in dogs (Gillen et al., 1988) and following subtotal oesophagectomy in humans where the gastric cardia is resected (Dresner et al., 2003). In the second case Barrett's could be explained by proximal migration of gastric body epithelium rather than gastric cardia epithelium and this theory would perhaps be better described as gastric migration with subsequent intestinal metaplasia.

2. Conversion of the stem cells of the oesophageal squamous epithelium

This theory has been the pre-eminent one for many years (Oh and deMeester, 2010). It suggests that the stem cells of the oesophagus undergo a metaplasia from a squamous to a columnar phenotype. Despite the predominant nature of this theory over recent years there is surprising little hard evidence to support the hypothesis.

The metaplasia process is believed to occur as a result of the chronic inflammation and damage associated with duodeno-gastro-oesophageal reflux. *In vitro* experiments using cell culture models and biopsy specimens have suggested that chronic exposure of oesophageal cells to acid and/or bile can result in the induction of genes associated with differentiation to an intestinal phenotype (Liu et al., 2006a, Hu et al., 2007). Experiments of this nature have not however demonstrated that goblet cells can be produced in this manner, merely that cells can demonstrate upregulation of genes and gene products associated with goblet cells. Animal experiments where oesophago-duodenostomy or oesophago-jejunostomy have been used to induce reflux into the oesophagus have shown that Barrett's like epithelium can develop above such an anastomosis (Melo et al., 1999, Pera et al., 1989, Clark et al., 1994). Whilst these experiments have resulted in the presence of goblet cells in the oesophagus they do not prove the oesophageal origin of these cells. It is possible that these result from proximal migration of duodenal or jejunal cells.

In addition whether this is situation is representative of the situation in humans remains open for debate (Nicholson et al., 2012).

3. Circulating bone marrow stem cells able to colonise a damaged oesophagus

Lethal irradiation of female rats followed by rescue with bone marrow from male donor rats allows the Y chromosome to be used as a marker for the fate of the transplanted bone marrow cells. Rats which have undergone this process followed by oesophagojejunostomy to induce reflux have been found to have both oesophageal squamous and columnar cells with a Y chromosome as the oesophagus heals (Sarosi et al., 2008). This suggests that cells from the bone marrow are able to migrate to the oesophagus and divide to produce more than one phenotype of cell to repair mucosal damage. Recently there have been some concerns that these results are inaccurate and that the presence of Y chromosomes in cells of the gastrointestinal tract may be the result of fusion between a bone marrow cell and a gastrointestinal (GI) cell rather than differentiation of a bone marrow cell to become a GI cell (Ferrand et al., 2011). The evidence for the role of bone marrow stem cells in the development of Barrett's oesophagus therefore remains highly speculative.

4. A residual population of embryonic cells that persist at the gastro-oesophageal junction

This theory has received much less attention than those outlined above. In the human embryo, the foregut is thought to arise from a common progenitor cell which expresses the p63 protein. In the mature oesophagus the cells of the superficial layers are p63 negative but it has been suggested that populations of embryonic type p63 positive cells persist at the squamo-columnar junction (Wang et al., 2011). In the presence of oesophageal damage the authors of this paper suggest that these embryonic cells are able to migrate towards the squamous epithelium and give rise to both squamous and columnar epithelial cells. As with other theories the evidence comes from animal models and is therefore subject to the usual concerns with regards to extrapolation to humans. This model is also unable to explain the occurrence of neo-Barrett's epithelium

after surgery where the proposed site of these embryonic cells has been removed.

5. Stem cells in oesophageal gland ducts

Submucosal glands are present in the wall of the human oesophagus. These glands are composed of individual mucus producing acini which lead into ducts which open onto the surface epithelium (Coad et al., 2005). Proximally these ducts are lined by columnar epithelium but the distal third is lined by squamous epithelium. This theory for the origin of Barrett's oesophagus proposes that these ducts contain multipotential stem cells (Barbera and Fitzgerald, 2010). Nicholson et al. (2012) used mitochondrial DNA mutations as clonal markers to study the development of Barrett's oesophagus. They found that oesophageal gland ducts can contain clonal patches of cells with a mitochondrial DNA mutation. This indicates a stem cell from which these mutant cells are derived should reside somewhere within the duct. The authors of this study were unable to identify a clonal mutation incorporating both columnar and squamous cells. Previous studies have however suggested that stem cells in either oesophageal ducts or glands are capable of producing both columnar and squamous epithelium (Leedham et al., 2008).

This theory would sit comfortably with evidence from the early animal studies where columnar metaplasia was observed to develop above a ring of intact squamous epithelium (Gillen et al., 1988). This suggested that Barrett's was not occurring as a result of proximal migration of columnar cells but that the cell of origin might lie within the oesophagus itself.

In addition to uncertainty about the cell of origin of Barrett's oesophagus the mechanism by which large areas of the oesophagus become lined with metaplastic epithelium is poorly understood. The selective 'sweep to fixation' model proposes that a single mutated stem cell with a selective advantage is able to clonally expand to fill an entire Barrett's segment (Maley et al., 2004). An alternative theory suggests that Barrett's oesophagus arises from multiple independent mutated clones (Leedham et al., 2008). According to this hypothesis there is no single founder mutation which is present throughout a

Barrett's segment. Instead mutations occur in multiple progenitor cells throughout the length of the oesophagus. These give rise to multiple distinct clones of metaplastic epithelium which then compete to colonise the oesophagus resulting in a mosaic pattern of clones across the segment.

Certain genetic mutations are known to occur relatively frequently in Barrett's oesophagus and oesophageal adenocarcinoma and are therefore useful targets to study. Mutations are thought to occur sequentially and to confer a biological advantage on the cells involved allowing them to grow and divide at an abnormal rate (Fitzgerald, 2006b). During the later stages of carcinogenesis genetic changes are believed to result in decreased apoptosis and increased angiogenesis allowing tumours to grow and metastasise. The presence, location and extent of spread of genetic mutations can be tracked within a segment of Barrett's oesophagus and this principle has been utilised to study the development and malignant progression of Barrett's oesophagus. Genes which have been widely studied in Barrett's oesophagus include *CDKN2A* which encodes for the p16 ink4a protein, *Tp53* which encodes for the p53 protein and *KRAS* which encodes for the Kras GTPase.

The *CDKN2A* (p16) gene acts as a cell cycle inhibitor and regulates the progression of cells from the first gap (G1) phase of the cell cycle through to the second DNA synthesis (S) phase. This is the major point of regulation for cell proliferation and the protein which acts as the master controller at this point is the retinoblastoma (Rb) protein (Wang and Souza, 2011). p16 inhibits cyclin D4/6 mediated phosphorylation of Rb preventing the cell proceeding through the cell cycle. Where mutation or methylation, results in inactivation of p16, cells are able to pass unhindered into the DNA synthesis (S) phase of the cell cycle and cell proliferation is increased.

The *TP53* gene is a tumour suppressor gene which has a central role in the induction of apoptosis, the process of programmed cell death which typically occurs after significant cellular damage has occurred (Wang and Souza, 2011, Meek, 2009). Like p16, p53 also has a role as an inhibitor of the cell cycle. In this respect it acts via target genes including p21 cyclin-dependent kinase inhibitor 1A (CDKN1A), 14-3-

3σ, and growth arrest and DNA damage-inducible gene (GADD45α) (Reinhardt and Schumacher, 2012). Loss of p53 consequently results in abnormalities of both the apoptosis pathway and the cell cycle.

The *KRAS* oncogene encodes for a protein which has a major role in cell proliferation. RAS-mediated signals regulate the function of proteins which promote passage from the G1 phase into the S phase of the cell cycle and proteins that influence apoptosis (Wang and Souza, 2011). KRAS is part of a kinase pathway which mediates cellular response to extracellular growth factors. This pathway is complex and it has been proposed that it is also disrupted by methods other that mutation of the *KRAS* gene (Jankowski et al., 1991). Mutation of the *KRAS* gene is rare in non-dysplastic Barrett's. In this early stage of Barrett's carcinogenesis, Ras pathway activation appears to occur as a result of increased levels of growth factors including epidermal growth factor receptor (EGFR) and transforming growth factor alpha (TGF-α). Increased levels of both of these growth factors have been found in biopsy samples of non-dysplastic Barrett's oesophagus (Jankowski et al., 1991). In the later stages of Barrett's carcinogenesis mutations of the *KRAS* gene become more common along with those of the *BRAF* gene which is part of the same pathway. Sommerer and colleagues (2004) found that around one third of Barrett's adenocarcinomas have either a *KRAS* or *BRAF* gene mutation.

Dysfunction of the RAS pathway is also a key factor in angiogenesis, the process by which cells are able to synthesise new blood vessels which is critical if tumours are to grow. The process is mediated via the Ras signalling pathway and is initiated by the binding of vascular endothelial growth factors (VEGFs) to their receptors (Wang and Souza, 2011).

Wong et al (2001) demonstrated that at least one CDKN2A allele was silenced either by mutation, loss of heterozygosity (LOH) or methylation in over 85% of patients with Barrett's oesophagus. They also found that mutated p16 glands underwent extensive clonal expansion to involve long (up to 17cm) segments of Barrett's oesophagus. The mechanism of expansion is unclear but gland fission (where one Barrett's gland bifurcates to form two daughter glands) has been shown to occur in Barrett's (Nicholson et al., 2012). Galipeau and colleagues

(1999) similarly demonstrated a high prevalence of abnormalities of both the p16 and p53 tumour suppressor genes in patients with Barrett's oesophagus with high grade dysplasia. Again these abnormalities were found to occupy long segments of the Barrett's oesophagus in some cases. More recent work by Leedham et al (2008) has suggested that Barrett's oesophagus comprises multiple independent clones (each with independent mutation profiles indicating a separate ancestry) rather than being the result of the expansion of a single clone. Marked genetic heterogeneity was observed between different glands of samples from the same patients.

In the context of neo-Barrett's oesophagus, these theories about the origins of metaplasia and malignant progression are particularly interesting. At the time of surgery these patients appear to have normal mucosa in the oesophageal remnant (Personal observations L Dunn and A D Burt). Despite this, a high proportion proceed to develop metaplasia over a relatively short period of time (D'journo et al., 2009, Dresner SM and Griffin SM, 2000). This raises the question of whether this simply reflects the high frequency and severity of reflux in this group or if there is an underlying susceptibility of the oesophageal epithelium to develop metaplasia and possibly subsequent dysplasia.

The theory of field cancerisation has been gaining credence over recent years. Initially proposed as a mechanism for the development of tumours of the oral squamous epithelium, this theory suggests that there can be widespread replacement of the normal cell population of an epithelium by a histologically non-dysplastic mutant clone that is predisposed to tumour development (Slaughter et al., 1953). Tumours subsequently occur as a result of further mutation events within the mutated field. This theory therefore proposes that tumour development begins sometime before there are any overtly visible histological changes. Field cancerisation has been described in the lung (Franklin et al., 1997), colon (Galandiuk et al., 2012, Leedham et al., 2009, Nosho et al., 2009) and skin (Hafner et al., 2010) and there is increasing recognition that it may also occur in the oesophagus (Zeki et al., 2011). Potentially this process could contribute to the development of neo-Barrett's after subtotal oesophagectomy. Whilst the residual oesophageal epithelium appears endoscopically and histologically normal there may be residual genetic

changes present within the cells of this epithelium which result in a predisposition to the development of metaplasia.

The aim of this study was to look for evidence of field cancerisation in the context of neo-Barrett's. Tumours and adjacent Barrett's oesophagus from patients who subsequently developed neo-Barrett's were screened for genetic mutations. Where mutations were present the aim was to determine if these same mutations were present in the post operative neo-Barrett's.

6.2 Methods

Patients were eligible for inclusion in this study if they had samples of neo-Barrett's oesophagus taken at endoscopy under the terms of the ethical approval granted by the County Durham and Tees Valley 2 REC. This work was conducted as a collaborative project involving the author and Dr Stuart Macdonald's team at the Cancer Research UK laboratories in London. Laser capture microdissection and polymerase chain reaction and sequencing practical work were divided equally between the author and Dr Shabuddin Khan, a fellow research student.

6.2.1 Tissue

Paraffin-embedded oesophagectomy blocks from 10 patients were obtained from the pathology archives of the Royal Victoria Infirmary, Newcastle-uponTyne. Slides were reviewed by an expert gastrointestinal pathologist to identify representative areas of oesophageal adenocarcinoma and, where possible, co-existing Barrett's oesophagus from the same patient.

Serial sections were cut at 5μm thickness and one sample was stained with haematoxylin and eosin (H&E).

6.2.2 Macrodissection of Specimens

Suitable areas of oesophageal adenocarcinoma and Barrett's oesophagus were confirmed using the H&E slide. Serial sections containing these areas were dewaxed and rehydrated by soaking in xylene and decreasing concentrations of alcohol through to water. Following de-waxing the identified representative areas from 5 serial slides were macrodissected using a sterile 21-guage needle.

Scraped samples were incubated in 30 μl of proteinase K solution (Picopure, Arcturus Bioscience, Mt View, California, USA) at 65℃ overnight. Following incubation a 10 minute incubation at 95℃ was used to denature the proteinase K.

6.2.3 Screening polymerase chain reaction and sequencing

The macro-dissected specimens were screened for mutations in the mutable regions of *CDKN2A* (exon 2) and TP53 (exons 5 – 8). In the case of *KRAS* the

specimens were screened for mutations in the whole gene sequence as the gene is much smaller.
Primers were designed using the Primer 3 website http://frodo.wi.mit.edu/primer3/input.htm). A nested PCR protocol was used for each primer sequence. Briefly 1 µl of extracted DNA was added to a 25 µl PCR reaction mixture containing 0.25 µl each of forward and reverse gene specific primers, 0.5 µl dNTP (Life Science, Buckinghamshire, UK), 0.2 µl of Taq polymerase (Qiagen, Crawley, UK), 2 µl of PCR template along with magnesium chloride, Q solution (Qiagen, Crawley, UK) and water in the quantities outlined in appendix H. First round PCR was prepared in a UV hood to minimise contamination and then subjected to 37 cycles of denaturing, annealing and extension. Annealing temperatures are provided in appendix F. 1 µl of first round PCR product was then subjected to a second round of PCR. Details of individual primer reactions for this round are detailed in appendix I. To ensure successful amplification prior to sequencing, 2nd round PCR products were electrophoresed through a 1.5% agarose gel (Sigma, UK).

PCR products were sequenced using BigDye terminator cycle sequencing on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, California, USA). Sequences obtained were reviewed for the presence of mutations and directly compared to the ensemble database. Identified possible mutations were correlated against the COSMIC database of somatic mutations in cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Single nucleotide polymorphisms were excluded by sequencing DNA extracted from either muscle or distant stroma.

Specimens identified as having mutations on the initial screen were put forward for laser capture dissection and further analysis on a gland-by-gland basis to assess how widespread the mutation was within the specimen. Where a mutation was present in either the original tumour or Barrett's oesophagus, the neo-Barrett's specimen from the patient was screened for the presence of the same mutation. In this case the neo-Barrett's biopsy specimen was sectioned, microdissected and sequenced for the identified mutation using the techniques described above.

6.2.4 Laser capture microdissection

5µm serial sections were cut from blocks identified as having a mutation on the initial screen. Six serial sections were mounted onto P.A.L.M. membrane slides (P.A.L.M., Microlaser Technologies, Benried, Germany) which had been UV treated to minimise contamination. In addition a further serially cut section was mounted onto a standard slide and stained with H&E. The P.A.L.M. mounted sections were stained with methylene green which was applied for 30 seconds, rinsed with tap water and allowed to dry thoroughly.

Suitable individual crypts for dissection were identified using the H&E slide. Each selected crypt was dissected out using the P.A.L.M. system and catapulted onto adhesive capped eppendorfs which had been UV treated in preparation. The process was repeated for the same crypt on each of the remaining 5 serial slides. Once the final sample had been catapulted, 14 µl of proteinase K solution (Picopure, Life Technologies) was added to the combined samples.

Eppendorfs were centrifuged at 3000rpm for 30 seconds, sealed with paraffin film and incubated at 65°C overnight. Following overnight incubation, a 10 minute incubation at 95°C was used to denature the proteinase K. Extracted DNA was then subjected to PCR sequencing as described above.

6.3 Results

6.3.1 Oesophagectomy samples

Samples were available from 10 patients as detailed below.

Paired Tumour and Barrett's oesophagus samples	6 patients
Tumour sample only	3 patients
Barrett's oesophagus only	1 patient

The samples of Barrett's oesophagus were obtained from the same en-bloc oesophagectomy specimen as the samples of tumour but from an area not exhibiting frank malignancy.

Analysis of the initial screening of the macro-dissected specimens revealed mutations in the samples from 4 patients (table 6.1). In each of these four cases the mutation was present in the specimen of adenocarcinoma. In one case the same mutation was present in both the sample of adenocarcinoma and the sample of Barrett's oesophagus.

Patient	Specimen type	*TP53* Exon-5	*TP53* Exon-6	*TP53* Exon-7	*TP53* Exon-8	*KRAS*	*CDKN2A* Exon-2
1	ACA	WT	WT	WT	WT	WT	WT
	BO	WT	WT	WT	WT	WT	WT
2	ACA	WT	WT	WT	WT	c.35G>A	WT
	BO	WT	WT	WT	WT	WT	WT
3	ACA	WT	WT	WT	WT	WT	WT
	BO	WT	WT	WT	WT	WT	WT
4	ACA	WT	WT	WT	WT	WT	WT
5	ACA	WT	WT	WT	WT	WT	WT
6	ACA	WT	WT	c.743G>A	WT	WT	WT
7	ACA	WT	WT	WT	WT	WT	WT
	BO	WT	WT	WT	WT	WT	WT
8	ACA	WT	WT	WT	WT	WT	WT
9	ACA	WT	WT	WT	WT	WT	c.238C>T
	BO	WT	WT	WT	WT	WT	WT
10	ACA	c.451C>T	WT	WT	WT	WT	WT
	BO	c.451C>T	WT	WT	WT	WT	WT

Table 6.1: Results of initial screen of macro-dissected oesophagectomy specimens (ACA – Adenocarcinoma, BO – Barrett's oesophagus, WT – Wild type).

6.3.2 Post oesophagectomy neo-Barretts biopsies

Neo-Barrett's biopsies from the 4 patients with an identified mutation in their resection specimen showed no evidence of this mutation. In addition no new mutation was identified in any of the studied exons, all were wild type.

6.3.3 Laser capture microdissection

The four samples found to contain mutations were subjected to laser capture microdissection of individual crypts with the intention of determining how widespread the identified mutation was within the tissue. Only exons identified as containing mutations in the original screening process were sequenced in this case.

Patient 2

Eight areas were dissected from this sample (figure 6.1). The *KRAS* mutation identified in the initial macrodissection was present in every microdissected sample.

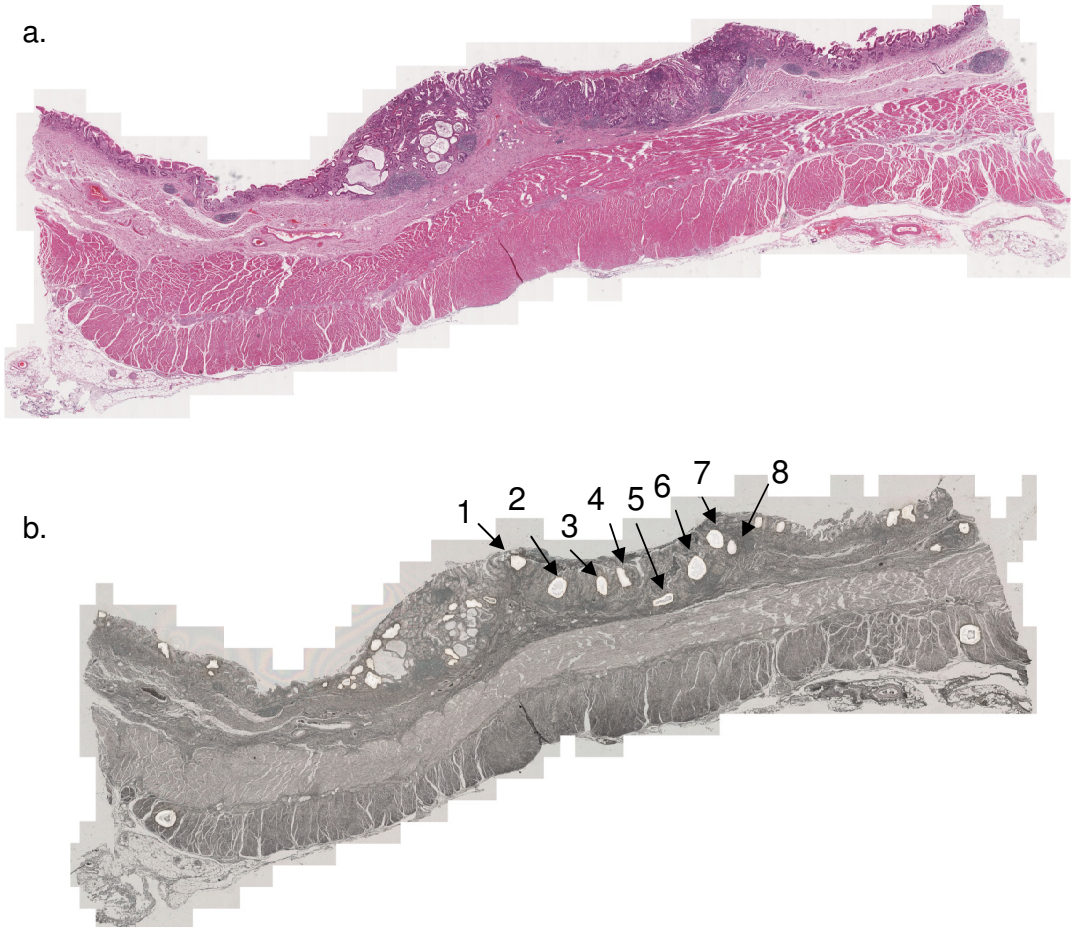


Figure 6.1: Specimen from patient 2 stained with (a) Haematoxylin and Eosin and (b) methylene green showing laser captured areas

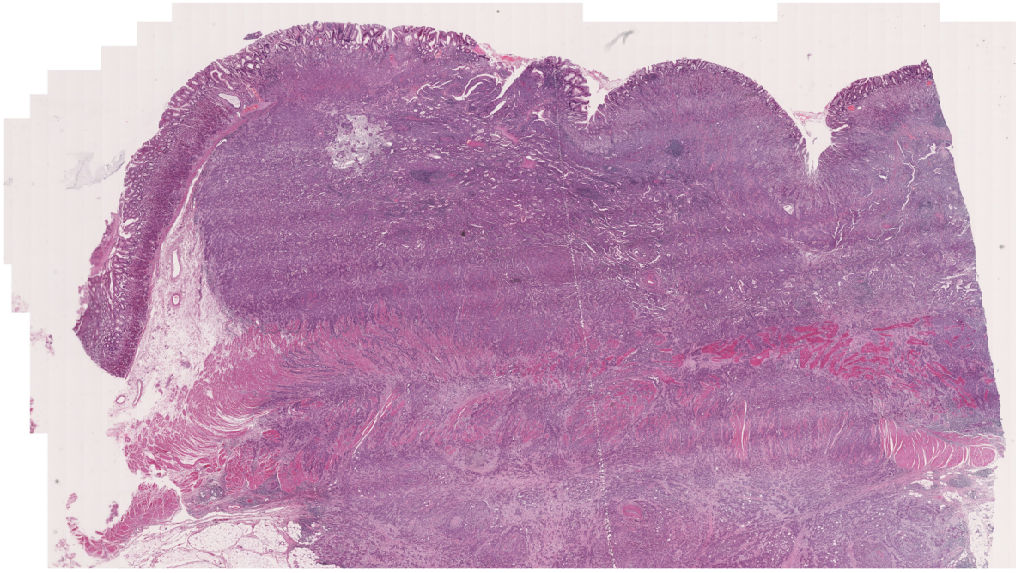
Dissected area	Tissue phenotype	*KRAS*
1	Adenocarcinoma	c.35G>A
2	Adenocarcinoma	c.35G>A
3	Adenocarcinoma	c.35G>A
4	Adenocarcinoma	c.35G>A
5	Adenocarcinoma	c.35G>A
6	Adenocarcinoma	c.35G>A
7	Adenocarcinoma	c.35G>A
8	Adenocarcinoma	c.35G>A

Table 6.3: Results of sequencing of laser captured areas of oesophagectomy block from patient 2 (WT – Wild type)

Patient 6

Five areas were dissected (figure 6.2). Areas 3 and 5 were from deeper within the tissue. The mutation identified in the initial macrodissection was a c.743G>A substitution in exon 7 of *TP53*.

a.



b.

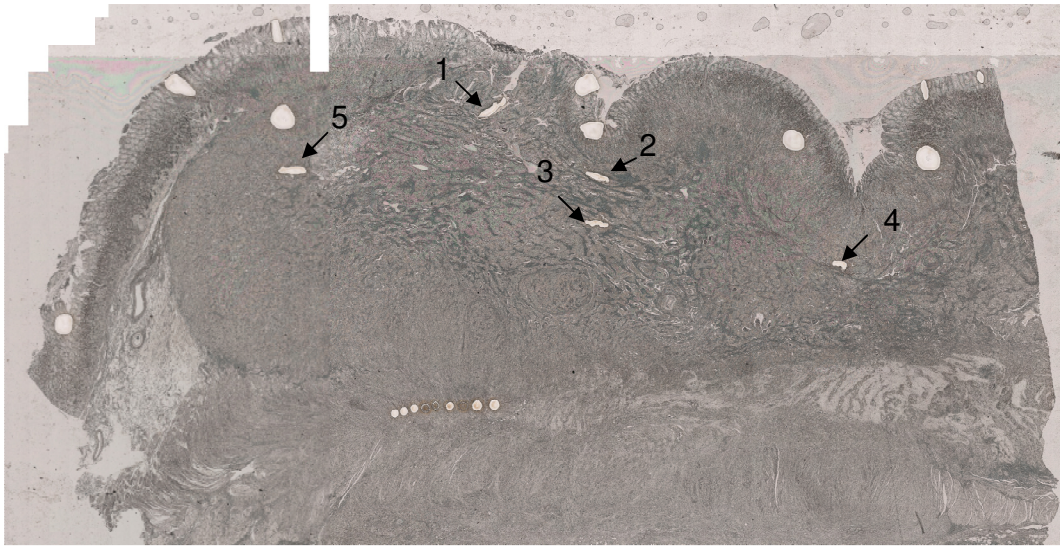


Figure 6.2: Specimen from patient 6 stained with (a) Haematoxylin and Eosin and (b) methylene green showing laser captured areas

Dissected area	Tissue phenotype	*TP53* Exon 7
1	Adenocarcinoma	c.743G>A
2	Adenocarcinoma	c.743G>A
3	Stroma	WT
4	Adenocarcinoma	c.743G>A
5	Stroma	WT

Table 6.4: Results of sequencing of laser captured areas of oesophagectomy block from patient 6 (WT – Wild type)

Patient 9

Five areas were dissected, four areas from the malignant epithelium and one from the adjacent squamous epithelium. The c.238C>T substitution in *CDKN2A* exon 2 was present in all of the areas of malignant epithelium which were microdissected. The squamous mucosa microdissected was wild type.

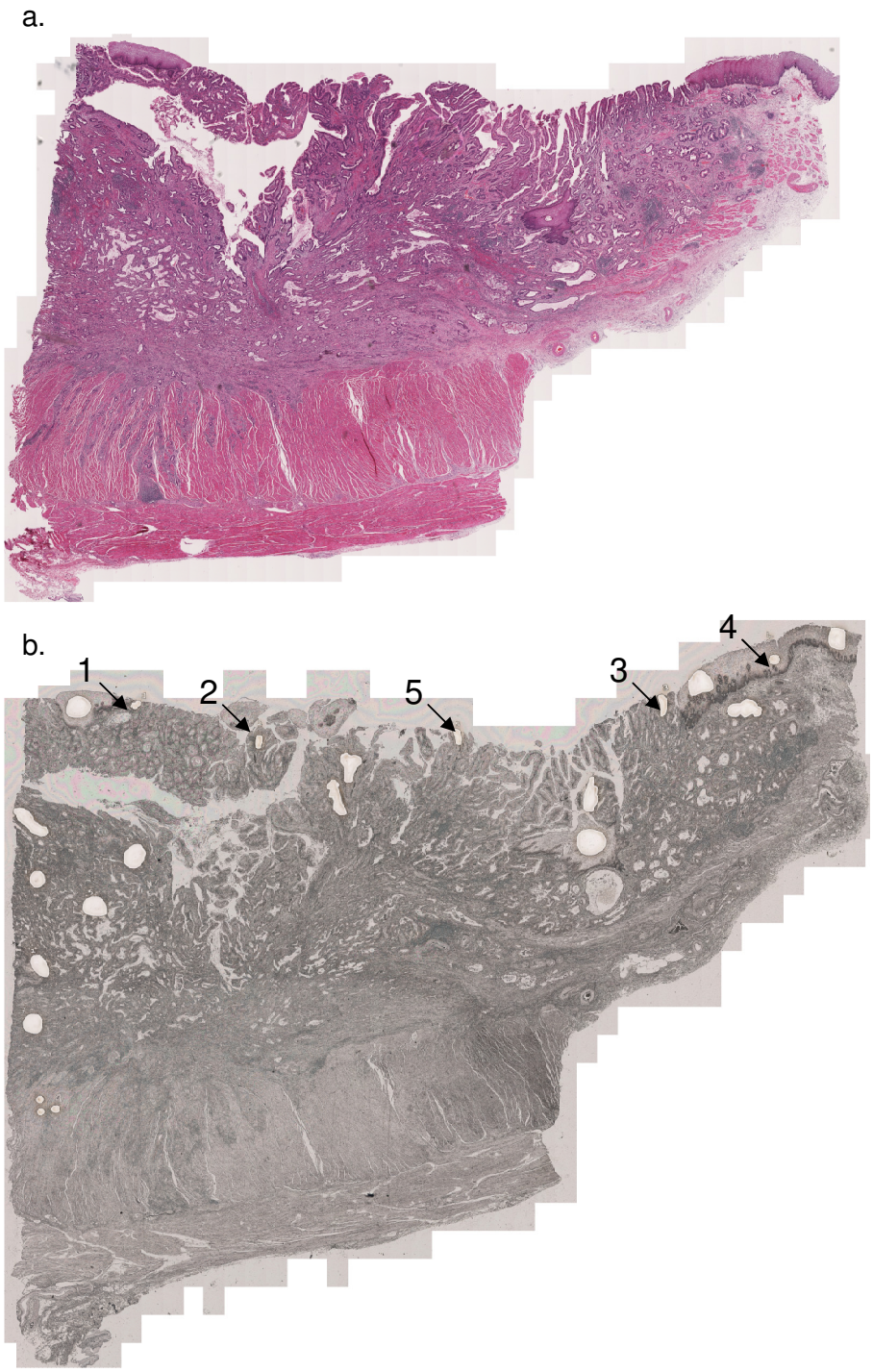


Figure 6.3: Specimen from patient 9 stained with (a) Haematoxylin and Eosin and (b) methylene green showing laser captured areas

Dissected area	Tissue phenotype	*CDKN2A* Exon 2
1	Adenocarcinoma	c.238C>T
2	Adenocarcinoma	c.238C>T
3	Adenocarcinoma	c.238C>T
4	Squamous	WT
5	Adenocarcinoma	c.238C>T

Table 6.5: Results of sequencing of laser captured areas of oesophagectomy block from patient 9 (WT – Wild type)

Patient 10

Nine areas were dissected, areas 1-7 were taken from the malignant epithelium and areas 8 and 9 were from the area of adjacent squamous epithelium contained within the same block. The mutation identified in the initial macrodissection was a c.451C>T in *TP53*.

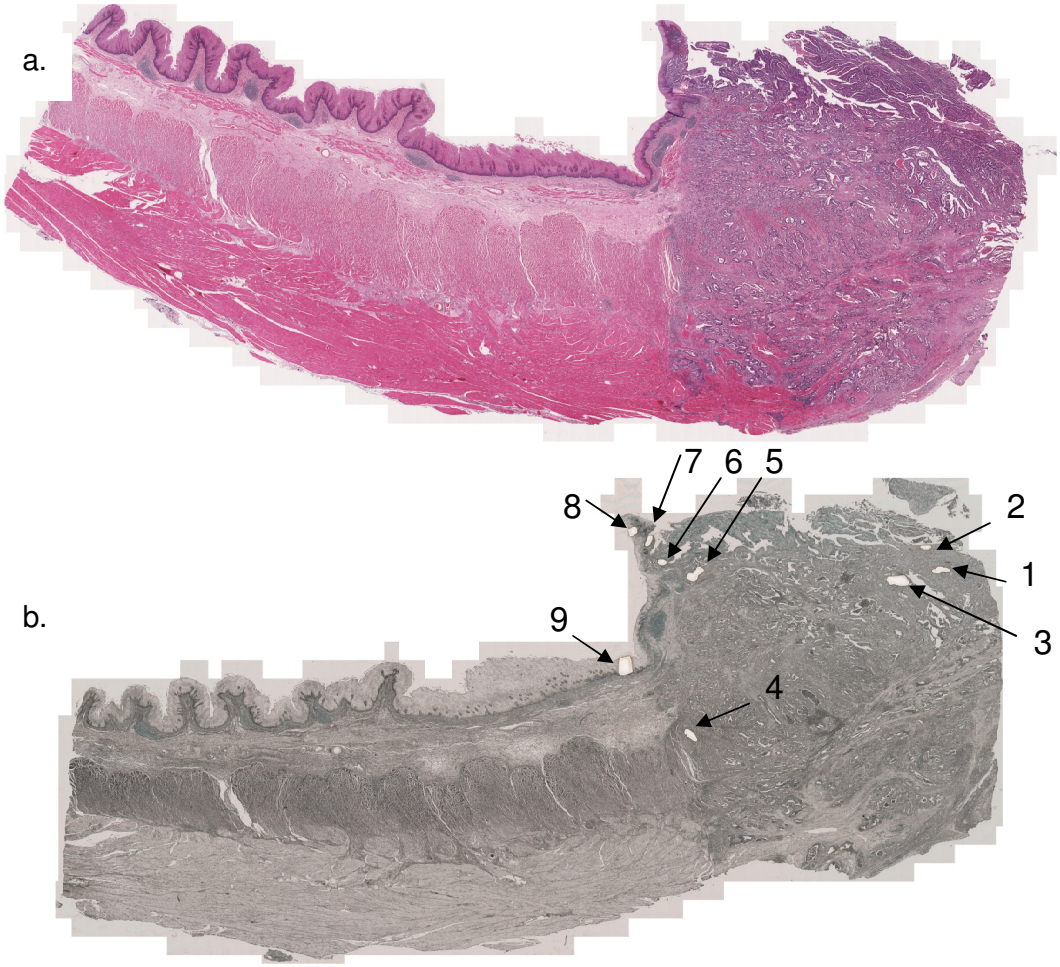


Figure 6.4: Specimen from patient 10 stained with (a) Haematoxylin and Eosin and (b) methylene green showing laser captured areas

Dissected area	Tissue phenotype	*TP53* Exon 5
1	Adenocarcinoma	c.451C>T
2	Adenocarcinoma	c.451C>T
3	Adenocarcinoma	c.451C>T
4	Adenocarcinoma	c.451C>T
5	Adenocarcinoma	c.451C>T
6	Adenocarcinoma	c.451C>T
7	Adenocarcinoma	c.451C>T
8	Squamous	WT
9	Squamous	WT

Table 6.6: Results of sequencing of laser captured areas of oesophagectomy block from patient 10 (WT – Wild type)

6.4 Discussion

The origins of neo-Barrett's are unknown. It is a common condition in post-oesophagectomy patients but we do not know if it is a de novo lesion or whether it arises from an unresected area of the original Barrett's. The resection margins are typically sufficient for all salmon-pink mucosa (the macroscopic phenotype of Barrett's) to be removed however, field cancerisation (the process by which an epithelial field becomes predisposed to cancer development) frequently is indistinguishable from non-cancerised areas (Slaughter et al., 1953). It is therefore important to screen neo-Barrett's and the original oesophagectomy in an attempt to ascertain putative genetic relationships between the two.

There is some evidence to suggest that mutated progenitor cells which are able to give rise to Barrett's metaplasia are also able to give rise to apparently normal squamous epithelium. Paulson and colleagues (2006) studied 20 patients who had islands of neo-squamous epithelium which had appeared within segments of Barrett's oesophagus following either medical or surgical acid reducing treatment. All patients included in this study had evidence of a clonally expanded population of cells with a mutation of either the *CDKN2A* or *TP53* genes. In 19 cases the neo-squamous epithelium was wild type but in one case there was evidence of the same *CDKN2A* mutation in the both the Barrett's oesophagus and the neo-squamous epithelium. The authors propose that this is evidence of a common precursor cell that is capable of generating both types of epithelia. In the context of neo-Barrett's the same process might occur in reverse, a mutated precursor cell in the proximal oesophagus might be giving rise to squamous epithelium at the time of surgery but following surgery when this area is exposed to reflux, this precursor cell might begin to produce neo-Barrett's epithelium. In this case one would expect there to be a clonal link between the neo-Barrett's epithelium and any Barrett's oesophagus present at the time of surgery.

In the present study, samples were initially macrodissected and known 'hotspots' in genes commonly mutated in oesophageal adenocarcinoma were sequenced. Macrodissection of either a whole biopsy specimen or of a relevant section of an oesophagectomy block allows an effective screening process for

genetic mutations to be undertaken. This process is relatively crude but it is time efficient. Where mutations are detected, samples can be subjected to further, more detailed analysis but in the relatively high number of samples where there are no mutations detected, time and reagents are not wasted by microdissecting and sequencing multiple individual crypts.

The limitation of the macrodissection screening process is that low copy number mutations can be missed either due to contamination by wild type stromal tissue or wild type adjacent crypts. Effectively the predominant wild type tissue masks a mutation only present in a tiny proportion of the sequenced tissue. In the current study a single *KRAS* mutation was detected in one sample using the macrodissection process but subsequent laser capture work by colleagues using the same original specimen revealed a mutation in exon 5 of *TP53* which was not evident in the macrodissected specimen even when sequencing was repeated.

The present study did not sequence the entire coding sequence of *TP53* and *CDKN2A* but instead sequenced selected exons. It is well recognised that the majority of mutations in these genes occur in certain 'hotspots' of the gene, exons 5-8 in the case of *TP53* and exon 2 in the case of *CDKN2A*. By sequencing these known 'hotspots' one would expect to pick up the majority of mutations which might be present in these genes. Whilst mutations in other parts of the gene would be missed using this approach the number of these is likely to be extremely small. The gene for *KRAS* is much smaller and it is possible to sequence the whole gene using a single round of PCR and it is therefore unnecessary to select mutation hotspots.

In the current study genetic mutations were found in samples from 4 out of 10 patients (40%). One sample contained a mutation of the *KRAS* gene, one sample contained a mutation of *CDKN2A* and 2 samples contained mutations of *TP53*. Analysis of the COSMIC database of somatic mutations in cancer suggests that 9% of oesophageal adenocarcinoma specimens contain a *KRAS* mutation, 10% contain a *CDKN2A* mutation and 51% contain a *TP53* mutation. The incidence of mutations detected in this study are therefore similar to what

would be expected for both *KRAS* and *CDKN2A* but lower than might be expected for *TP53*.

Patients were included in the present study as they had gone on to develop neo-Barrett's oesophagus. Schneider and colleagues (2000) detected *TP53* mutations in 30 of 59 samples taken from patients with oesophageal adenocarcinoma. They found that tumours with mutations of this gene were associated with a markedly poorer prognosis compared those without. In the present study using samples from patients who had survived long enough to develop neo-Barrett's there is likely to have been a selection bias towards patients with a better prognosis who potentially are less likely to have had tumours with *TP53* mutations. With such small numbers of patients, the lower than expected incidence of *TP53* mutations observed in this study could be entirely due to chance.

Laser capture microdissection allows specimens to be analysed on a crypt by crypt basis. This technique can be used to study clonality of tissues as it is possible to reveal mutations present within single crypts or small patches of tissue which would be masked by the predominant wild type tissue if a whole biopsy or oesophagectomy block specimen were used. In a tissue of monoclonal origin the tissue should be genotypically, as well as phenotypically homogenous.

Laser capture microdissection and sequencing of samples from the four patients with genetic mutations in the present study demonstrated that the observed mutation was present in all crypts dissected from the adenocarcinoma epithelium. This supports the theory that adenocarcinomas are monoclonal. In one case the mutation found in the adenocarcinoma was also present in an area of Barrett's oesophagus present within the same oesophagectomy specimen. This provides a putative link between the two suggesting that the tumour arose from this surrounding Barrett's metaplasia. Two further patients with identifiable mutations in their adenocarcinoma also had areas of Barrett's available to study from the same oesophagectomy specimen. In neither of these cases was the mutation detectable within the Barrett's specimen. In these cases no link between the Barrett's oesophagus and the co-existing

adenocarcinoma was demonstrable. An important consideration here is the fact that the Barrett's oesophagus resection blocks were taken from the same oesophagectomy specimens but not necessarily from areas adjacent to the tumours. We know that Barrett's oesophagus is heterogeneous and it may be that these tumours arose from surrounding Barrett's oesophagus but not necessarily from the area captured in our specimen block.

Leedham et al (2008) pioneered the use of the techniques described above in the study of sporadic Barrett's oesophagus. They dissected individual crypts from Barrett's oesophagus biopsies and from oesophagectomy blocks and analysed them for both point mutations, as undertaken in the present study and for loss of heterozygosity of the tumour suppressor genes *CDKN2A*, *TP53* and *APC* (adenomatous polyposis coli). In this study it was observed that both single biopsies and resection specimens of Barrett's oesophagus exhibited genetic heterogeneity. The authors suggested a new model for the clonal evolution of Barrett's oesophagus whereby mutations occur in multiple progenitor cells. Each of these mutated progenitor cells gives rise to a separate clone of cells and these evolve and compete resulting in a heterogeneous mosaic within a Barrett's oesophagus segment. Some clones may have a selective advantage and consequently expand more widely however there is no such thing as an underlying 'founder mutation' present throughout a Barrett's oesophagus.

The present study suggests that whilst Barrett's oesophagus metaplasia may be polyclonal in origin, adenocarcinoma arising within Barrett's oesophagus is monoclonal. None of the mutations detected within the adenocarcinoma specimens were detected in the samples of neo-Barrett's oesophagus from the same patients.

There are two potential explanations for the development of neo-Barrett's metaplasia in the oesophageal remnant following sub-total oesophagectomy. The first theory is that this is spontaneous metaplasia occurring as a result of the profound reflux of acid and bile into the oesophageal remnant following surgery. According to this theory, neo-Barrett's would therefore develop in much the same fashion as sporadic Barrett's oesophagus. A second theory is

that patients with oesophageal adenocarcinoma have an underlying field change present within, and throughout the oesophageal epithelium which renders the epithelium unstable and prone to the development of metaplasia and subsequent dysplasia. The present study found no evidence to suggest that mutations present in the original tumours were present within the oesophageal remnant and therefore found no evidence to support the theory of a field change present throughout the oesophagus.

Whilst there is no data to support a theory of oesophageal field change, the present study provides insufficient data to exclude this possibility. The mutations detected in the original specimens were detected in areas of oesophageal adenocarcinoma and not within adjacent areas of non-neoplastic tissue as determined by laser capture microdissection. These mutations may therefore occur late in the metaplasia-dysplasia-adenocarcinoma sequence. The post oesophagectomy samples studied did not include any areas of dysplasia or adenocarcinoma and this may explain the absence of the mutations observed in the oesophagectomy specimens. It could be argued that there may be an undetected persisting mutation, or loss of heterozygosity which predisposes to the earliest stages in the conversion of oesophageal squamous epithelium to metaplastic columnar epithelium and which underlies both the development of the original carcinoma and the subsequent neo-Barrett's oesophagus.

Chapter 7. Summary and Discussion

This study sought to evaluate the incidence of columnar metaplasia in the oesophageal remnant following subtotal oesophagectomy and reconstruction with a gastric conduit. Small studies have suggested that this is a common phenomenon occurring in over a third of patients but the present study is the largest to address this issue and confirms the high prevalence.

Columnar metaplasia occurred with a similar incidence following surgery for both oesophageal adenocarcinoma and squamous cell carcinoma inferring that it occurs de novo following surgery rather than being due to residual disease or an underlying predisposition of the patient to develop Barrett's oesophagus. Previous studies within the Northern Oesophago-gastric cancer unit have demonstrated profound reflux of both acid and bile into the oesophageal remnant following oesophagectomy and it is suggested that this is the causative mechanism for the development of this neo-Barrett's metaplasia (Dresner et al., 2003).

The earliest case of columnar metaplasia in this study was observed only 9 months following surgery. This finding suggests that the initial conversion to a columnar mucosa can occur much more quickly than has been believed. While patients with Barrett's oesophagus often have a long history of symptomatic reflux, it appears that given the right conditions, oesophageal squamous mucosa can transform into a metaplastic columnar epithelium after only a few months exposure to the injurious effects of duodeno-gastric reflux. Gastro oesophageal reflux disease (GORD) represents a significant health problem in the Western world in particular, with an estimated prevalence of 10 – 20% defined by weekly heartburn and/or acid regurgitation (Dent et al., 2005). The vast majority of patients with GORD will have no long term sequelae but the association with Barrett's oesophagus and oesophageal adenocarcinoma is a source of concern for health care professionals. Investigation, with the gold standard test being gastroscopy, is relatively expensive and invasive and methods to risk stratify those patients at high risk of developing significant pathology are urgently required. Guidelines from the National Institute for health and clinical excellence (2004) recommend referral for assessment only for patients over the age of 55 who have unexplained or persistent symptoms or dyspepsia associated with alarm symptoms. The present study casts some

doubt on the theory that complications of GORD only occur in the context of longstanding symptoms and suggests that duration of symptoms may not be as significant as previously thought in terms of risk stratification of patients.

One hypothesis of the present study was that neo-Barrett's metaplasia occurring in the oesophageal remnant is an inevitable consequence of subtotal oesophagectomy and reconstruction with a gastric conduit. This hypothesis is not proven. The prevalence of columnar metaplasia increased with time from surgery and was over two thirds for patients in whom over eight years had elapsed since surgery. Whether the remaining patients would develop neo-Barrett's metaplasia over a longer period of time is unclear. The present study does however confirm that neo-Barrett's can be expected to develop in almost all long term survivors following oesophagectomy. The other important inference of the present study is that the vast majority of individuals have the potential to develop Barrett's oesophagus in the presence of significant reflux disease.

Recent developments in endoscopic techniques have provided less invasive options for the treatment of early oesophageal carcinoma in the form of endoscopic mucosal resection. For younger patients one concern has been whether further invasive carcinomas may develop as the oesophagus remains in-situ following treatment. Subtotal oesophagectomy has been suggested as the treatment of choice for these patients as it removes the vulnerable tissue and prevents any future neoplasia which may be associated with recurrence or field changes. The results of the present study suggest that this group of young patients with early disease and a good prognosis are at high risk of developing columnar metaplasia in the oesophageal remnant over a period of years. Surgery perhaps does not offer the absolute guarantee against recurrent adenocarcinoma that these patients seek. Critically, there were no cases of dysplasia or adenocarcinoma within the oesophageal remnant of the cohort of patients in the present study. Any risk of this appears small but there is clearly a degree of concern given the high prevalence and apparent rapid development of the metaplastic epithelium.

In the United Kingdom the vast majority of patients undergoing oesophagectomy do so for malignant disease. The median age at the time of surgery was 67 in 2009, the latest year for which figures are available.(2010) The majority of patients also have relatively advanced disease (stage 2 or 3) and will unfortunately die as a result of their disease despite undergoing surgery with curative intent. As a result of these factors the overall 5 year survival following oesophagectomy remains significantly under 50% (Dresner SM and Griffin SM, 2000). The absence of any cases of dysplasia in this cohort of 126 patients is very reassuring. The median follow up period was 3.6 years and the cohort included 47 patients in whom more than five years had elapsed since the time of surgery. These findings suggest that the majority of patients undergoing oesophagectomy in the United Kingdom are at very low risk of developing dysplasia or adenocarcinoma in their oesophageal remnant during their normal lifespan and a finding of neo-Barrett's is not clinically significant for these individuals.

For certain subgroups of patients the propensity to develop neo-Barrett's may be much more relevant. Patients undergoing surgery for early stage malignant disease have a good chance of cure and long term survival. Paediatric patients undergoing surgery for intractable corrosive stricture or oesophageal atresia may have a similar reconstruction and in other parts of the world oesophagectomy for end stage achalasia remains relatively common. All of these patients might be expected to live for many decades after surgery and the risk of malignant progression within neo-Barrett's epithelium may be much more significant.

There are no national guidelines on endoscopic follow up of patients following oesophagectomy. The finding of a high prevalence of neo-Barrett's in a previous study in the Northern Oesophago-gastric Cancer Unit (Dresner et al., 2003) led to the introduction of routine endoscopic follow up in the unit as outlined in Chapter 2. On the basis of the present larger study, with a significant cohort of long term survivors it would appear that this approach is unnecessarily cautious. There appears to be no justification for early routine endoscopy at one year. The policy of routine endoscopy at five years is more controversial. Certainly this seems unlikely to detect dysplasia or

adenocarcinoma but it could be argued that this is an appropriate time to screen for neo-Barrett's particularly in younger patients who may benefit from long-term endoscopic follow up. Clearly patient co-morbidities at this time also need to be taken into account. Paediatric patients are an important consideration, they may be at the highest risk of malignant progression in neo-Barrett's due to their long post operative life expectancy. Follow up of these individuals may be disrupted when they reach adolescence and transfer to adult services. The present study does not address the issue of neo-Barrett's in paediatric patients but this has been reported elsewhere and endoscopic follow up is recommended by some surgeons (Lindahl et al., 1990, Borgnon et al., 2004).

The present study observed that a greater period of time since surgery had elapsed for patients with columnar epithelium demonstrating specialised intestinal metaplasia compared to those with other forms of columnar epithelium. These observations suggest that the first step in the metaplasia-dysplasia-adenocarcinoma sequence is conversion to a non-intestinalised columnar epithelium. There has been much debate about the importance of specialised intestinal metaplasia in making the diagnosis of Barrett's oesophagus and a lack of concordance remains between guidelines. North American guidelines do not recognise columnar epithelium without specialised intestinal metaplasia as Barrett's oesophagus. UK guidelines do recognise non-intestinalised columnar epithelium as Barrett's oesophagus, providing the location of the biopsies is confirmed as being oesophageal in origin. In post surgical patients it is much easier to identify the gastro-oesophageal junction and the location of biopsies within the oesophagus is easier to confirm. The present study suggests that patients with cardiac or fundic types of metaplasia may simply be at an early stage of the disease process and that these epithelia are part of the oesophageal metaplasia sequence rather than a result of sampling error and inadvertent gastric biopsy. This finding has potentially important clinical implications. Patients without intestinal metaplasia are unlikely to be offered surveillance and follow up in the United States as they do not meet the diagnostic criteria for Barrett's oesophagus. The present study suggests that these patients may well go on to develop intestinal metaplasia but they are likely to have been discharged from clinical follow up.

Surveillance programmes for Barrett's oesophagus are common but there is limited evidence to support their efficacy in reducing mortality from oesophageal cancer. The UK based Barrett's Oesophagus Surveillance Study (BOSS) aims to assess the efficacy of surveillance. This randomised controlled trial will compare regular endoscopic surveillance with endoscopy at need for the prevention of early mortality from oesophageal adenocarcinoma (Jankowski and Barr, 1996). The study is not due to report for several years but if surveillance is shown to confer a survival advantage the issue of how to accurately diagnose Barrett's will become even more important. The present study would suggest that patients without intestinal metaplasia should be included although, as this appears to represent earlier stage disease, longer surveillance intervals might be appropriate.

Studying the development of Barrett's oesophagus represents a significant clinical problem. It is not possible to accurately identify patients at high risk from their demographic profile or symptoms. The incidence of oesophageal adenocarcinoma has increased dramatically over the last three decades and despite improvements in chemotherapy regimes and surgical outcomes the associated mortality remains high. It seems likely that real improvements in the mortality associated with this condition will come as a result of prevention or detection of early disease rather than improvements in the treatment of advanced disease. Barrett's oesophagus is recognised as the major risk factor for oesophageal adenocarcinoma and the prevention of this condition or the modification of its malignant potential are key research targets. Over recent years many potential chemopreventive agents and predictive markers of cancer progression have been identified but in order to test these a robust high throughput model for the development and progression of Barrett's is required (Attwood et al., 2008). Models allow pre-clinical testing of potential markers and treatments without the expense and protracted time periods associated with standard trials in humans. Compounds which show promise in model based studies can be fast tracked to human studies and resources allocated appropriately.

The present study confirms that columnar metaplasia develops in a high proportion of patients following subtotal oesophagectomy in an accelerated fashion. These patients represent a possible human model in which pharmacological agents which might provide protection from Barrett's could undergo preliminary testing. If this post-surgical model is compared against the characteristics of an ideal Barrett's model outlined in Chapter 1 some of the potential benefits above animal and cell culture models are obvious.

1. Spontaneous metaplasia should occur in the model species

Criteria is fulfilled

2. Model species should be genetically similar to man

Criteria is fulfilled

3. Model should include a lifelike model of reflux

Criteria is partially fulfilled. Reflux tends to be more severe than in non-surgical patients and bile reflux is more significant given the pyloroplasty.

4. Model should represent the complex confounding factors in humans e.g. ageing, lifestyle factors and genetic heterogeneity

Criteria is probably fulfilled. Ageing and lifestyle factors are similar in post surgical patients compared to others. The cohort in the present study included a high proportion of patients with adenocarcinoma and it is possible that these individuals are not truly genetically representative of the population as a whole.

The absence of any cases of dysplasia in this study suggests that post-oesophagectomy patients are unlikely to be a good model for the malignant progression of Barrett's oesophagus. Other studies suggest that progression to dysplasia and adenocarcinoma does occur but the numbers involved are thankfully small and the timescales appear to be long. There therefore appears to be no advantage in using these 'model' patients as opposed to patients with

sporadically occurring Barrett's oesophagus. The value of the post-oesophagectomy patient model appears be in demonstrating the development rather than progression of columnar metaplasia.

Cellular protein expression as determined by immunohistochemistry allows for more detailed examination and comparisons of epithelia than is possible with conventional histopathology alone. Cellular proteins have been studied in Barrett's oesophagus by a number of research groups and the present study aimed to ascertain whether post-oesophagectomy neo-Barrett's tissue is characterised by the same molecular markers. Clearly for post-oesophagectomy patients to be considered as a model for the development of Barrett's oesophagus it is essential that the columnar metaplasia occurring in this situation closely resembles that which occurs spontaneously in the general population.

The present study demonstrated the recognised 'classical Barrett's staining pattern' for cytokeratins 7 and 20 in over two thirds of neo-Barrett's samples, a prevalence which is within the range reported by those investigating sporadic Barrett's oesophagus. In a subgroup of patients with neo-Barrett's oesophagus with specialised intestinal metaplasia the prevalence of this classical pattern was even higher at more than 80%. This finding provides further support for the theory that intestinalised columnar epithelium represents a more advanced stage of metaplasia. The present study also demonstrated the presence of neuroendocrine differentiation in neo-Barrett's tissue in the form of Chromogranin A positive cells. This feature is known to occur commonly in sporadic Barrett's oesophagus and was universal in the neo-Barrett's samples studied.

When considering studies of Barrett's oesophagus it is important to take into account the fact that many different definitions have been used over the years and this is likely to contribute to the marked variations in prevalence of molecular markers reported. The results in the present study do not provide conclusive proof that post-oesophagectomy neo-Barrett's is identical to sporadic Barrett's oesophagus but they are certainly similar. This provides further evidence for the theory that this is an appropriate model for the early stages in

the development of Barrett's oesophagus with markers known to be expressed in Barrett's oesophagus frequently present. There is therefore, now reasonable evidence that neo-Barrett's is endoscopically, microscopically and molecularly similar to sporadic Barrett's oesophagus.

There are drawbacks to the post-oesophagectomy human model for the development of Barrett's oesophagus. Overall long-term survival after oesophagectomy is poor due to the high prevalence of advanced stage disease and the average age of patients. Under 1200 patients are recorded as having undergone oesophagectomy in England and Wales in 2009 according to a national audit (National Oesophago-gastric Cancer Audit, 2010) and the number of patients available for long term follow up is therefore small. Patients who have undergone this type of surgery have often undergone significant physical and emotional stress and enrolling them in studies of neo-Barrett's might cause unnecessary anxiety. This issue would need to be carefully considered by researchers and ethics committees particularly given that the present study suggests that neo-Barrett's follows a fairly indolent clinical course for these individuals.

The present study also demonstrated the presence of trefoil proteins in neo-Barrett's tissue. This is the first time that these proteins have been studied in neo-Barrettt's tissue. All three recognised trefoil proteins were expressed to varying extents and in a similar pattern to that reported in sporadic Barrett's oesophagus. Median time from surgery was greater in those patients who demonstrated TFF3 expression compared to those who did not express this protein suggesting that this trefoil may not be present in very early stage Barrett's oesophagus but becomes more prevalent over time. Serial biopsies from post-oesophagectomy patients would be required to prove this temporal association but ethical concerns mean this type of study is unlikely to be undertaken.

TFF3 has been proposed as a biomarker which could be used to screen for Barrett's oesophagus. There are ongoing clinical trials investigating non-endoscopic screening for Barrett's oesophagus using a sponge capsule to capture oesophageal cells (Kadri SR et al., 2010). TFF3 staining is used in this

study to identify Barrett's cells with a sensitivity and specificity of over 90% for what are deemed clinically relevant segments of 2cm or more. The results of the present study support the use of TFF3 as a biomarker for Barrett's oesophagus but also raise the possibility that early stage disease could be missed by this technique if TFF3 has not yet become established in the new segment.

Endoscopic screening for Barrett's oesophagus has been practiced by some clinicians, particularly in the United States (Rubenstein et al., 2008). There is no good evidence to support this approach however and it is not currently recommended by either American or British guidelines. At present the only routinely available method of screening for Barrett's is endoscopy. This is expensive, invasive and carries small but not insignificant risks. Oesophageal adenocarcinoma remains relatively rare with less that 6000 cases per year in England and Wales. It is not possible to accurately identify high risk patients as gastro-oesophageal reflux is so common and up to 40% of patients with adenocarcinoma do not report chronic reflux symptoms (Lagergren et al., 1999). Those who undertake screening for Barrett's argue that it potentially identifies individuals who might benefit from surveillance to detect progression to adenocarcinoma at an early, treatable stage. Coupled with the lack of evidence for Barrett's oesophagus surveillance it is unlikely that current methods of screening are ever likely to be recommended.

The ongoing work investigating screening with a sponge capsule and TFF3 as a biomarker is interesting as it has the potential to change the cost and clinical effectiveness balance in favour of screening and surveillance. The test is easier to administer and cheaper than endoscopy and used in combination with other biomarkers it might have a role in surveillance. If new, non-surgical treatments for early oesophageal cancer, ablative treatments for Barrett's and chemotherapeutic agents fulfil their promise in long term studies we may in future be looking to a situation where we have a cost effective test for an easily modifiable condition. This situation is undoubtedly some way off but is not beyond the realms of possibility. In this context a biomarker to identify patients with Barrett's oesophagus becomes critically important.

Oesophageal adenocarcinoma, which develops from Barrett's oesophagus is one of the most lethal malignancies and its incidence is increasing rapidly. Future research is needed both in terms of the basic and clinical sciences in order to better identify patients at risk, to understand and potentially modify the development and progression of the metaplasia-dysplasia-adenocarcinoma sequence and to treat patients with invasive adenocarcinoma. The human model for the development of Barrett's oesophagus provided by post-oesophagectomy patients is not without limitations but may go some way to help with this process.

Appendices

Appendix A. Ethical Approval

National Research Ethics Service
County Durham & Tees Valley 2 Research Ethics Committee
Professorial Unit of Surgery
University Hospital of North Tees
Piperknowle Road
Stockton-on-Tees
TS19 8PE

Telephone: 01642 624164
Facsimile: 01642 624164

31 July 2008

Professor S Michael Griffin
Professor of Gastrointestinal Surgery
Northern Oesophagogastric Unit, Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP

Dear Professor Griffin

Full title of study: **Molecular and genetic changes associated with the development of Barrett's metaplasia following oesophagectomy**
REC reference number: **08/H0908/25**

Thank you for your letter of 28 July, responding to the Committee's request for further information on the above research, subject to the conditions specified below.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA. There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.5	31 March 2008
Investigator CV		
Protocol	1	25 March 2008
Covering Letter		25 April 2008
Letter of invitation to participant	1	19 May 2008
Participant Information Sheet: Re:addtional biopsy at endoscopy	2	19 May 2008
Participant Information Sheet: RE: additional tests on stored samples	2	19 May 2008
CV for Lorna Dunn		25 March 2008
Letter of support From Professor Sloan regarding transportation of samples		22 July 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0908/25	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Leigh Pollard

pp **Rachel Duncan**
Chair

Email: leigh.pollard@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Ms Amanda Tortice, Clinical Research Facility, Royal Victoria Infirmary, Newcastle upon Tyne*

Appendix B – Patient Information leaflet

The Newcastle upon Tyne Hospitals NHS Trust

Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP

Patient Information Sheet and Consent Form

Patient Initials: **Subject Number:**

Study Title: **Molecular and genetic changes in Barrett's oesophagus following surgery**

Name of Researchers: Professor Michael Griffin, Dr. Lorna Dunn

You are being asked to allow the research team to undertake additional laboratory tests on samples of tissue which were stored at the time of your operation and at your endoscopy tests. Before you decide if you are willing to take part, it is important for you to understand the background to this project. Therefore please take time to read the following information carefully and discuss it with others if you wish.

Ask us if there is anything that is not clear or if you would like more information. Take as much time as you want to decide whether or not you wish to take part.

Thank you for reading this.

What is the background and purpose of the study?

Barrett's oesophagus is a condition where the lining of the lower end of the gullet (oesophagus) is transformed from its normal lining to one which has a more complex nature, similar in many ways to the lining of the stomach. It is believed this condition is caused by acid from the stomach spilling back up into the oesophagus (gastro-oesophageal reflux) over many years. Some patients, including you, develop Barrett's in the remaining part of their oesophagus following surgery to remove the rest of the oesophagus.

Many questions remain unanswered about Barrett's oesophagus. The process by which the normal lining of the oesophagus changes over time is not understood. It is also unclear why some patients develop Barrett's after their surgery whilst others do not.

The study which we are performing will hopefully clarify some of the confusion in Barrett's oesophagus, in particular, Barrett's which occurs following surgery. We are seeking volunteers to help us in this study and invite you to take part. Our plan is to carry out additional laboratory tests on samples of tissue which were routinely stored at the time of your surgery and following your more recent endoscopy tests. These specimens will be analysed under the microscope using techniques which are not used routinely but which have proved useful in other studies. By comparing the findings between different groups of patients we hope to gain a greater understanding of how Barrett's develops.

Why have I been chosen?

You have been asked to consider taking part in this study because you have had an oesophagectomy and have gone on to develop Barrett's changes in the remaining part of your oesophagus.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you should keep this information sheet to keep and sign and return the consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide not to take part in this study you will not be disadvantaged and your medical treatment and care will not be changed in any way.

What will happen to me if I take part?

The research team will obtain your old tissue samples and carry out some additional laboratory tests on them. You will not need to undergo any additional tests or procedures or attend any additional appointments.

What are the possible benefits of taking part?

This study will not directly help you but could lead to more appropriate treatments for future generations of patients with the same condition. The extra tests we plan to carry out are for research purposes only and will not affect the care you require or receive in the future.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Contact 0191 2829697). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure

Complaints can be sent to:
The Complaints Officer, Freeman Hospital, Newcastle. NE7 7DN

Who has reviewed the study?
This study has been reviewed by the County Durham and Tees Valley 2 Research Ethics Committee

Contact Details:

For further information about the study you can speak to the Clinical Research Fellow:

Dr. Lorna Dunn Tel: 0191 2829697

Thank you for taking the time to read this information sheet. If you wish to participate in this study please complete and return the attached consent form.

Appendix C. Protocol for processing and paraffin embedding of biopsy specimens

1. Specimens transferred in 10% neutral buffered formalin
2. Formalin washed from tissue with tap water
3. Tissue transferred to 70% ethanol in distilled water for 24 hours
4. Tissue passed through two changes of 70% ethanol for one hour each
5. Tissue transferred to 80% ethanol in distilled water for 2 hours
6. Tissue transferred to 90% ethanol in distilled water for 2 hours
7. Tissue passed through 3 changes of 100% ethanol each for 2 hours
8. Tissue transferred to isopropyl alcohol for 2 hours
9. Tissue transferred to 1:1 isopropyl alcohol:chloroform each for 2 hours
10. Tissue passed through 2 changes of chloroform each for 2 hours
11. Tissue passed through 2 changes of molten paraffin wax each for 3 hours
12. Paraffin wax block allowed to cool embedding tissue

Appendix D. Protocol for section cutting and de-waxing and rehydrating

Cutting

1. Paraffin block allowed to cool on ice for 10-15 minutes
2. Block locked in position on microtome and surface apposed to blade
3. 15µm sections trimmed from block until surface of tissue exposed
4. 5 µm sections cut
5. Sections floated and flattened on surface of water bath at 40℃
6. Sections retrieved onto glass slides and allowed to drain
7. Sections dried in oven at 60℃ for 45 minutes
8. Sections allowed to cool

Dewaxing and Rehydrating

Procedure undertaken using Leica Autostainer XL

1. Sections heated in oven at 65℃ for 15 minutes
2. Sections soaked in xylene for 1 minute
3. Sections soaked in 2 further changes of xylene for 30 seconds each
4. Sections soaked in 2 changes of absolute alcohol for 30 seconds each
5. Sections soaked in 95% alcohol for 1 minute
6. Sections washed in distilled water for 1 minute

Appendix E. Immunohistochemistry staining protocol for Cytokeratins 7 and 20

Protocol for use with the BenchMark XT automated staining system (Ventana, Roche diagnostics, Sussex, UK)

1. ***** Select EZ Prep *****
2. ***** Start Timed Steps *****
3. ***** Mixers Off *****
4. Warm up Slide to 75 Deg C, and incubate for 4 Minutes
5. Apply EZ Prep Volume Adjust (Ventana, Tucson, Arizona, USA)
6. Rinse slide
7. Apply EZ Prep Volume Adjust
8. Rinse slide
9. Apply EZ Prep Volume Adjust
10. Apply Liquid Coverslip (Ventana, Tucson, Arizona, USA)
11. Warmup Slide to 76 Deg C, and incubate for 4 minutes
12. Rinse slide
13. Apply Depar Volume Adjust (Ventana, Tucson, Arizona, USA)
14. Apply Liquid Coverslip
15. Disable slide heater
16. ***** Mixers On *****
17. [Short - 8 Minute Conditioning]
18. Rinse slide
19. Apply Cell Conditioner #1 (Ventana, Tucson, Arizona, USA)
20. Apply Liquid Coverslip
21. ***** Select SSC Wash *****
22. Warm up slide to 95 Deg C, and incubate for 8 Minutes
23. [Mild - 30 Minute Conditioning]
24. Apply Cell Conditioner #1
25. Apply Liquid Coverslip
26. Warmup Slide to 100 Deg C, and incubate for 4 minutes
27. Apply Liquid Coverslip
28. Apply Cell Conditioner #1
29. Apply Liquid Coverslip
30. Apply Cell Conditioner #1

31. Apply Liquid Coverslip
32. Apply Cell Conditioner #1
33. Apply Liquid Coverslip
34. Apply Cell Conditioner #1
35. Apply Liquid Coverslip
36. Apply Cell Conditioner #1
37. Apply Liquid Coverslip
38. [Standard - 60 Minute Conditioning]
39. Apply Cell Conditioner #1
40. Apply Liquid Coverslip
41. Apply Cell Conditioner #1
42. Apply Liquid Coverslip
43. Apply Cell Conditioner #1
44. Apply Liquid Coverslip
45. Apply Cell Conditioner #1
46. Apply Liquid Coverslip
47. Apply Cell Conditioner #1
48. Apply Liquid Coverslip
49. Apply Cell Conditioner #1
50. Apply Liquid Coverslip
51. Apply Cell Conditioner #1
52. Apply Liquid Coverslip
53. Disable slide heater
54. Incubate for 8 minutes
55. Rinse slide With Reaction Buffer (Ventana, Tucson, Arizona, USA)
56. Adjust slide volume with Reaction Buffer
57. Apply Liquid Coverslip
58. Rinse slide with Reaction Buffer
59. Adjust slide volume with Reaction Buffer
60. Apply Liquid Coverslip
61. ***** Procedure Synchronization *****
62. Warm up Slide to 37 Deg C, and incubate for 4 minutes
63. Rinse slide with Reaction Buffer
64. Adjust slide volume with Reaction Buffer

65. Apply One Drop of UV INHIBITOR (Ventana, Tucson, Arizona, USA) , Apply Liquid Coverslip, and incubate for 4 minutes
66. Rinse slide with Reaction Buffer
67. Adjust slide volume With Reaction Buffer
68. Apply Liquid Coverslip
69. Warm up slide to 37 Deg C, and incubate for 4 Minutes
70. Rinse Slide with Reaction Buffer
71. Adjust slide volume with Reaction Buffer
72. Apply Liquid Coverslip
73. Apply one drop of primary antibody and Incubate for 32 minutes
74. Rinse slide with Reaction Buffer
75. Adjust slide volume With Reaction Buffer
76. Apply Liquid Coverslip
77. Warm up Slide to 37 Deg C, and incubate for 4 minutes
78. Rinse slide with Reaction Buffer
79. Adjust slide volume with Reaction Buffer
80. Apply one drop of Ultraview horse radish peroxidase universal multimer (Ventana, Tucson, Arizona, USA), apply Liquid Coverslip, and incubate for 8 minutes
81. Rinse slide with Reaction Buffer
82. Adjust slide volume with Reaction Buffer
83. Apply Liquid Coverslip
84. Rinse slide with Reaction Buffer
85. Adjust slide volume with Reaction Buffer
86. Apply one drop of Ultraview DAB universal chromogen and one drop of Ultraview DAB H2O2 (both Ventana, Tucson, Arizona, USA), apply Liquid Coverslip, incubate for 8 Minutes
87. Rinse slide with Reaction Buffer
88. Adjust slide volume with Reaction Buffer
89. Apply One Drop of Ultraview copper, apply Liquid Coverslip, and incubate for 4 minutes
90. Rinse slide with Reaction Buffer
91. Adjust slide volume with Reaction Buffer
92. Apply one drop of Hematoxylin II counterstain (Ventana, Tucson, Arizona, USA), apply Liquid Coverslip and incubate for 8 Minutes

93. Rinse slide with Reaction Buffer
94. Adjust slide volume with Reaction Buffer
95. Apply Liquid Coverslip
96. Rinse slide with Reaction Buffer
97. Adjust slide volume with Reaction Buffer
98. Apply one drop of Bluing reagent (Ventana, Tucson, Arizona, USA), apply Liquid Coverslip and Incubate for 4 Minutes
99. Rinse slide with Reaction Buffer
100. Apply Liquid Coverslip
101. Disable slide heater
102. ***** Select Optional Wash *****
103. ***** Select SSC Wash *****
104. ***** Start Timed Steps *****
105. Rinse slide with Reaction Buffer

Appendix F. Immunohistochemistry staining protocol for chromogranin A

Protocol for use with the BenchMark XT automated staining system (Ventana, Roche diagnostics, Sussex, UK)

1. ***** Select EZ Prep *****
2. ***** Start Timed Steps *****
3. ***** Mixers Off *****
4. Warm up slide to 75 Deg C, and incubate for 4 minutes
5. Apply EZ Prep Volume Adjust (Ventana, Tucson, Arizona, USA)
6. Rinse slide
7. Apply EZ Prep Volume Adjust
8. Rinse slide
9. Apply EZ Prep Volume Adjust
10. Apply Liquid Coverslip (Ventana, Tucson, Arizona, USA)
11. Warm up slide to 76 Deg C and incubate for 4 minutes
12. Rinse slide
13. Apply Depar Volume Adjust (Ventana, Tucson, Arizona, USA)
14. Apply Liquid Coverslip
15. Disable slide heater
16. ***** Mixers On *****
17. [Short - 8 Minute Conditioning]
18. Rinse slide
19. Apply Cell Conditioner #1 (Ventana, Tucson, Arizona, USA)
20. Apply Liquid Coverslip
21. ***** Select SSC Wash *****
22. Warm up slide to 95 Deg C and incubate for 8 minutes
23. [Mild - 30 Minute Conditioning]
24. Apply Cell Conditioner #1
25. Apply Liquid Coverslip
26. Warm up slide to 100 Deg C and incubate for 4 minutes
27. Apply Liquid Coverslip
28. Apply Cell Conditioner #1
29. Apply Liquid Coverslip
30. Apply Cell Conditioner #1

31. Apply Liquid Coverslip
32. Apply Cell Conditioner #1
33. Apply Liquid Coverslip
34. Apply Cell Conditioner #1
35. Apply Liquid Coverslip
36. Apply Cell Conditioner #1
37. Apply Liquid Coverslip
38. Disable slide heater
39. Incubate for 8 minutes
40. Rinse slide with Reaction Buffer
41. Adjust slide volume with Reaction Buffer
42. Apply Liquid Coverslip
43. Rinse slide with Reaction Buffer
44. Adjust slide volume with Reaction Buffer
45. Apply Liquid Coverslip
46. ***** Procedure Synchronization *****
47. Warm up Slide to 37 Deg C and incubate for 4 minutes
48. Rinse slide with Reaction Buffer
49. Adjust slide volume with Reaction Buffer
50. Apply one drop of ultraView Inhibitor (Ventana, Tucson, Arizona, USA), apply Liquid Coverslip, and incubate for 4 minutes
51. Rinse slide with Reaction Buffer
52. Adjust slide volume with Reaction Buffer
53. Apply Liquid Coverslip
54. Warm up slide to 37 Deg C and incubate for 4 minutes
55. Rinse slide with Reaction Buffer
56. Adjust slide volume with Reaction Buffer
57. Apply Liquid Coverslip
58. Apply one drop of primary antibody and incubate for 32 minutes
59. Rinse slide with Reaction Buffer
60. Adjust slide volume with Reaction Buffer
61. Apply Liquid Coverslip
62. Warm up slide to 37 Deg C and incubate for 4 minutes
63. Rinse slide with Reaction Buffer
64. Adjust slide volume with Reaction Buffer

65. Apply one drop of ultraView horseradish peroxidise universal multimer, apply Liquid Coverslip and Incubate for 8 minutes
66. Rinse slide with Reaction Buffer
67. Adjust slide volume with Reaction Buffer
68. Apply Liquid Coverslip
69. Rinse slide with Reaction Buffer
70. Adjust slide volume with Reaction Buffer
71. Apply one drop of ultraView DAB and one drop of ultraView DAB H2O2, apply Liquid Coverslip, incubate for 8 minutes
72. Rinse slide with Reaction Buffer
73. Adjust slide volume with Reaction Buffer
74. Apply one drop of ultraView copper, apply Liquid Coverslip and incubate for 4 minutes
75. Rinse slide with Reaction Buffer
76. Adjust slide volume with Reaction Buffer
77. Apply one drop of Hematoxylin II counterstain (Ventana, Tucson, Arizona, USA), apply Coverslip, and incubate for 8 Minutes
78. Rinse slide with Reaction Buffer
79. Adjust slide volume with Reaction Buffer
80. Apply Liquid Coverslip
81. Rinse slide with Reaction Buffer
82. Adjust slide volume with Reaction Buffer
83. Apply one drop of Bluing reagent (Ventana, Tucson, Arizona, USA), apply Liquid Coverslip and incubate for 4 minutes
84. Rinse slide with Reaction Buffer
85. Apply Liquid Coverslip
86. Disable slide heater
87. ***** Select Optional Wash *****
88. ***** Select SSC Wash *****
89. ***** Start Timed Steps *****
90. Rinse slide with Reaction Buffer

Appendix G. Trefoil factor Immunohistochemistry Protocol

1. Sections prepared as described
2. Endogenous peroxidase activity blocked by soaking in methanol-hydrogen peroxide for 10 mins
3. Slides washed in tris-buffered saline (TBS) for 3 x 5 minutes
4. Antigen retrieval by heating slides in citrate buffer (pH6) in a pressure cooker for 1 minute once full pressure reached.
5. Slides cooled in running tap water
6. Slides incubated with avidin/biotin blocking kit (Vector Labs Inc, Ca, USA) according to manufacturers instructions.
7. Slides washed in TBS for 3 x 5 minutes
8. Slides incubated with normal rabbit serum for 10 minutes
9. Primary antibodies diluted with normal rabbit serum as follows

TFF1	1:10
TFF2	1:1000
TFF3	1:20

10. Slides incubated with primary antibodies at room temperature for 1 hour
11. Slides washed in TBS for 3 x 5 minutes
12. Slides incubated with bitinylated goat anti-mouse secondary antibody (Vector Labs Inc, Ca, USA) for 1 hour.
13. Slides washed in TBS for 3 x 5 minutes
14. Slides incubated with avidin-biotin peroxidase complex (Vectastain ABC Kit, Vector Labs Inc, Ca, USA) for 30 minutes
15. Slides washed in TBS for 3 x 5 minutes
16. Slides stained with 3,3'-diaminobenzidine/hydrogen peroxide
17. Slides washed in running tap water
18. Slides counterstained by dipping in haematoxylin for 15 seconds
19. Slides rinsed in running tap water
20. Slides soaked in Scott's tap water for 30 seconds
21. Slides rinsed in running tap water
22. Slides dehydrated by immersion in a series of increasing concentrations of ethanol, 50%, 75%, 95%, 100%, 100%.
23. Slides cleared in xylene
24. Sections mounted under cover slips

Appendix H. First round PCR reagent quantities and annealing temperatures

Primer	***$MgCl_2$***	***Q Solution***	***Annealing temperature***
TP53 Exon-5	1 µl	5 µl	55℃
TP53 Exon-6	2 µl	5 µl	60℃
TP53 Exon-7	1 µl	5 µl	60℃
TP53 Exon-8	2 µl	5 µl	60℃
KRAS	2 µl	5 µl	60℃
CDKN2A Exon-2	2 µl	5 µl	60℃

Appendix I. Second round PCR reagent quantities and annealing temperatures

Primer	***$MgCl_2$***	***Q Solution***	***Annealing temperature***
TP53 Exon-5	1 µl	5 µl	60℃
TP53 Exon-6	1 µl	0 µl	60℃
TP53 Exon-7	1 µl	5 µl	60℃
TP53 Exon-8	2 µl	0 µl	60℃
KRAS	2 µl	5 µl	55℃
CDKN2A Exon-2	2 µl	5 µl	60℃

References

AHNEN, D., POULSOM, R., STAMP, G., ELIA, G., PIKE, C., JEFFERY, R., LONGCROFT, J., RIO, M., CHAMBON, P. & WRIGHT, N. (1994) The ulceration-associated cell lineage (UACL) reiterates the Brunner's gland differentiation programme but acquires the proliferative organization of the gastric gland. *The Journal of Pathology,* 173**,** 317-26.

ALFARO, L., BERMAS, H., FENOGLIO, M., PARKER, R. & JANIK, J. (2005) Are patients who have had a tracheoesophageal fistula repair during infancy at risk for esophageal adenocarcinoma during adulthood. *Journal of Pediatric Surgery,* 40**,** 719-20.

ALLISON, P. & JOHNSTONE, A. (1953) The oesophagus lined with gastric mucous membrane. *Thorax,* 8**,** 87-101.

ALY, A. & JAMIESON, G. (2004) Reflux after oesophagectomy. *British Journal of Surgery,* 91**,** 137-141.

ANDERSON, L., WATSON, R., MURPHY, S., JOHNSTON, B., COMBER, H., MCGUIGAN, J., REYNOLDS, J. & MURRAY, L. (2007) Risk factors for Barrett's oesophagus and oesophageal adenocarcinoma: Results from the FINBAR study. *World Journal of Gastroenterology,* 13**,** 1585-1594.

ANDERSON, L. A., MURRAY, L. J., MURPHY, S. J., FITZPATRICK, D. A., JOHNSTON, B. T., WATSON, R. G. P., MCCARRON, P. & GAVIN, A. T. (2003) Mortality in Barrett's oesophagus: results from a population based study. *Gut,* 52**,** 1081-1084.

ARUL GS, P. D. (2008) Oesophageal replacement in children. *Annals of the Royal College of Surgeons of England,* 90**,** 7-12.

ATTWOOD, S., HARRISON, L.-A., PRESTON, S. L. & JANKOWSKI, J. A. (2008) Esophageal Adenocarcinoma in "Mice and Men": Back to Basics! *American Journal of Gastroenterology,* 103**,** 2367-2372.

BAKKE, I., QVIGSTAD, G., BRENNA, E., SANDVIK, A. & WALDUM, H. (2000) Gastrin has a specific porliferative effect on the rat enterochromaffin-like cell, but not on the parietal cell: a study by elutriation centrifugation. *Acta Physiologica Scandinavica,* 169**,** 29-37.

BARAK, V., GOIKE, H., PANARETAKIS, K. & EINARSSON, R. (2004) Clinical utility of cytokeratins as tumour markers. *Clinical Biochemistry,* 37**,** 529-540.

BARBERA, M. & FITZGERALD, R. (2010) Cellular origin of Barrett's metaplasia and oesophageal stem cells. *Biochemical society transactions,* 38**,** 370-373.

BARRETT, N. (1950) Chronic peptic ulcer of the oesophagus and 'oesophagitis'. *British Journal of Surgery,* 38**,** 175-82.

BARRETT, N. (1957) The lower esophagus lined by columnar epithelium. *Surgery,* 41**,** 881-94.

BAX, D., SIERSEMA, P., MOONS, L., VAN DEKKEN, H., TILANUS, H. W., KUSTERS, J. & KUIPERS, E. J. (2007) CDX2 Expression in Columnar Metaplasia of the Remnant Esophagus in Patients Who Underwent Esophagectomy. *Journal of Clinical Gastroenterology,* 41**,** 375-379.

BERSENTES, K., FASS, R., PADDA, S., JOHNSON, C. & SAMPLINER, R. E. (1998) Prevalence of Barrett's Esophagus in Hispanics Is Similar to Caucasians. *Digestive Diseases and Sciences,* 43**,** 1038-1041.

BOLLSCHWEILER, E., WOLFGARTEN, E., GUTSCHOW, C. & HOLSCHER, A. (2001) Demographic Variations in the Rising Incidence of Esophageal Adenocarcinoma in White Males. *Cancer,* 92**,** 549-55.

BORGNON, J., TOUNIAN, P., AUBER, F., LARROQUET, M., BOERIS CLEMEN, F., GIRARDET, J. & AUDRY, G. (2004) Esophageal replacement in children by an isoperistaltic gastric tube: a 12-year experience. *Pediatric Surgery International,* 20**,** 829-833.

BREMNER, C., LYNCH, V. & ELLIS, F. (1970) Barrett's esophagus: congenital or acquired? An experimental study of esophageal mucosal regeneration in the dog. *Surgery,* 68**,** 209-16.

BREMNER, C., LYNCH, V. & ELLIS, F. (1978) Barrett's esophagus: congenital or acquired? An experimental study of esophageal mucosal regeneration in the dog. *Surgery,* 68**,** 209-26.

BRUNNER, G., ATHMANN, C. & SCHNEIDER, A. (2012) Long-term, open-label trial: safety and efficacy of continuous maintenance treatment with pantoprazole for up to 15 years in severe acid-peptic disease. *Alimentary Pharmacology & Therapeutics,* 36**,** 37-47.

BUTTAR, N., WANG, K., SEBO, T., RIEHLE, D., KRISHNADATH, K., LUTZKE, L., ANDERSON, M., PETTERSON, T. & BURGART, L. (2001) Extent of high-grade dysplasia in Barrett's esophagus correlates with risk of adenocarcinoma. *Gastroenterology,* 120**,** 1630-1639.

CAMERON, A. & LOMBOY, C. (1992) Barrett's Esophagus: Age, Prevalence, and Extent of Columnar Epithelium. *Gastroenterology,* 103**,** 1241-1245.

CAMERON, A., ZINSMEISTER, A., BALLARD, D. & AL., E. (1990) Prevalence of columnar-lined Barrett's esophagus. Comparison of population-based clinical and autopsy findings. *Gastroenterology,* 99**,** 918-922.

CARNEY, C. N., ORLANDO, R. C., POWELL, D. W. & DOTSON, M. M. (1981) Morphologic alterations in early acid-induced epithelial injury of the rabbit esophagus. *Laboratory Investigation,* 45**,** 198-208.

CAYGILL, C., WATSON, A., REED, P. & HILL, M. (2003) Characteristics and regional variations of patients with Barrett's oesophagus in the UK. *European Journal of Gastroenterology and Hepatology,* 15**,** 1217-1222.

CHANDRASOMA, P. (2005) Controversies of the cardiac mucosa and Barrett's oesophagus. *Histopathology,* 46**,** 361-373.

CHANDRASOMA, P., LOKUHETTY, D., DEMEESTER, T., BREMNER, C., PETERS, J., OBERG, S. & GROSHEN, S. (2000) Definition of histopathologic changes in gastroesophageal reflux disease. *American Journal of Surgical Pathology,* 24**,** 344-51.

CHANDRASOMA, P., WICKRAMASINGHE, K., MA, Y. & DEMEESTER, T. (2007) Is intestinal metaplasia a necessary precursor lesion for adenocarcinomas of the distal esophagus, gastroesophageal junction and gastric cardia? *Diseases of the Esophagus,* 20**,** 36-41.

CHANG, C., LAO-SIRIEIX, P., SAVE, V., DE LA CUEVA MENDEZ, G., LASKEY, R. & FITZGERALD, R. (2007) Retinoic acid-induced glandular differentiation of the oesophagus. *Gut,* 56**,** 906-917.

CHANG, S., DOHRMAN, A., BASBAUM, C., HO, S., TSUDA, T., TORIBARA, N., GUM, J. & KIM, Y. (1994) Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. *Gastroenterology,* 107**,** 28-36.

CHAVES, P., DIAS PEREIRA, A., CRUZ, C., SUSPIRO, A., MENDES DE ALMEIDA, J., LEITAO, C. & SOARES, J. (2002) Recurrent columnar-lined esophageal segments - study of the phenotypic characteristics using intestinal markers. *Diseases of the Esophagus,* 15**,** 282-286.

CHU, P. & WEISS, L. (2002) Keratin expression in human tissues and neoplasms. *Histopathology,* 40**,** 403-39.

CLARK, G., SMYRK, T., MIRVISH, S., ANSELMINO, M., YAMASHITA, Y., HINDER, R., DEMEESTER, T. & BIRT, D. (1994) Effect of gastroduodenal juice and dietary fat on the development of Barrett's esophagus and esophageal neoplasia: an experimental rat model. *Annals of Surgical Oncology,* 1**,** 252-61.

COAD, R., WOODMAN, A., WARNER, P., BARR, H., WRIGHT, N. & SHEPHERD, N. (2005) On the histogenesis of Barrett's oesophagus and its associated squamous islands: a three-dimensional study of their morphological relationship with native oesophageal gland ducts. *The Journal of Pathology,* 206**,** 388-394.

CONIO, M., BLANCHI, S., LAPERTOSA, G., ROBERTO FERRARIS, R., SABLICH, R., MARCHI, S., D'ONOFRIO, V., LACCHIN, T., IAQUINTO, G., MISSALE, G., RAVELLI, P., CESTARI, R., BENEDETTI, G., MACRÌ, G., FIOCCA, R., MUNIZZI , F. & FILIBERTI, R. (2003) Long-term endoscopic surveillance of patients with Barrett's esophagus. incidence of dysplasia and adenocarcinoma: a prospective study. *American Journal of Gastroenterology,* 98**,** 1931-1939.

COOK, M., GREENWOOD, D., HARDIE, L., WILD, C. & FORMAN, D. (2008) A Systematic Review and Meta-Analysis of the Risk of Increasing Adiposity on Barrett's Esophagus. *American Journal of Gastroenterology,* 103**,** 292-300.

D'JOURNO, X., MARTIN, J., RAKOVICH, G., BRIGAND, C., GABOURY, L., FERRARO, P. & DURANCEAU, A. (2009) Mucosal Damage in the Esophageal Remnant After Esophagectomy and Gastric Transposition. *Annals of Surgery,* 249**,** 262-268.

DA ROCHA, J., RIBEIRO, U., SALLUM, R., SZACHNOWICZ, S. & CECCONELLO, I. (2008) Barrett's Esophagus (BE) and Carcinoma in the Esophageal Stump (ES) After Esophagectomy with Gastric Pull-Up in Achalasia Patients: A Study Based on 10 Years Follow-Up. *Annals of Surgical Oncology,* 15**,** 2903-2909.

DAS, D., CHILTON, A. & JANKOWSKI, J. (2009) Chemoprevention of oesophageal cancer and the AspECT trial. *Recent Results in Cancer Research,* 181**,** 161-9.

DE LEYN, P., COOSEMANS, W. & LERUT, T. (1992) Early and late functional results in patients with intrathoracic gastric replacement after

oesophagectomy for carcinoma. *European Journal of Cardiothoracic Surgery,* 6**,** 79-84.

DEMEESTER, S., WICKRAMASINGHE, K., LORD, R., FRIEDMAN, A., BALAJI, N., CHANDRASOMA, P., HAGEN, J., PETERS, J. & DEMEESTER, T. (2002) Cytokeratin and DAS-1 Immunostaining Reveal Similarities Among Cardiac Mucosa, CIM and Barrett's Esophagus. *American Journal of Gastroenterology,* 97**,** 2514-2523.

DENNIS, J., HVIDSTEN, T. & WIT, E. (2005) Markers of adenocarcinoma characteristic of the site of origin: development of a diagnostic algorithm. *Clinical Cancer Research,* 11**,** 3766-72.

DENT, J., EL-SERAG, H., WALLANDER, M. & JOHANSSON, S. (2005) Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut,* 54**,** 710-717.

DEURLOO, J., EKKELKAMP, S., TAMINIAU, J., KNEEPKENS, C., TEN KATE, F., BARTELSMAN, J., LEGEMATE, D. & ARONSON, D. (2005) Esophagitis and Barrett esophagus after correction of esophageal atresia. *Journal of Pediatric Surgery,* 40**,** 1227-31.

DEVESA, S., BLOT, W. & FRAUMENI, J. (1998) Changing Patterns in the Incidence of Esophageal and Gastric Carcinoma in the United States. *Cancer* 83**,** 2049-2053.

DI PIETRO M, PETERS CJ & FITZGERALD RC (2008) Clinical puzzle: Barrett's oesophagus. *Disease Models and Mechanisms,* 1**,** 26-31.

DIGNASS, A., LYNCH-DEVANEY, K., KINDON, H., THIM, L. & PODALSKY, D. (1994) Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *The Journal of Clinical Investigation,* 94**,** 376-83.

DOBHAN, R. & CASTELL, D. (1993) Normal and abnormal proximal esophageal acid exposure: results of ambulatory dual-probe pH monitoring. *American Journal of Gastroenterology,* 88**,** 25-9.

DRESNER, S., GRIFFIN, S., WAYMAN, J., BENNETT, M., HAYES, N. & RAIMES, S. (2003) Human model of duodenogastro-oesophageal reflux in the development of Barrett's metaplasia. *British Journal of Surgery,* 90**,** 1120-1128.

DRESNER SM & GRIFFIN SM (2000) Pattern of recurrence following radical oesophagectomy with two field lyphadenectomy. *British Journal of Surgery,* 87**,** 1426-1433.

DULAI, G., GUHA, S., KAHN, K., GORNBEIN, J. & WEINSTEIN, W. (2002) Preoperative prevalence of Barrett's esophagus in esophageal adenocarcinoma: a systematic review. *Gastroenterology,* 122**,** 26-33.

DUNN, L., ROBERTSON, A., IMMANUEL, A. & GRIFFIN, S. (2010) Barrett's Adenocarcinoma 52 Years After Subtotal Esophagectomy for Pediatric Peptic Stricture. *The Annals of Thoracic Surgery,* 89**,** 604-606.

EDA, A., OSAWA, H., SATOH, K., YANAKA, I., KIHIRA, K., ISHINO, Y., MUTOH, H. & SUGANO, K. (2003) Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *Journal of gastroenterology,* 38**,** 14-22.

EISEN, G., SANDLER, R., MURRAY, S. & GOTTFRIED, M. (1997) The relationship between gastroesophageal reflux disease and it's complications with Barrett's esophagus. *American Journal of Gastroenterology,* 92**,** 27-31.

EL-SERAG, H. (2008) Role of obesity in GORD-related disorders. *Gut,* 57**,** 281-284.

EL-ZIMAITY, H. & GRAHAM, D. (2001) Cytokeratin Subsets for Distinguishing Barrett's Esophagus From Intestinal Metaplasia in the Cardia Using Endoscopic Biopsy Specimens. *American Journal of Gastroenterology,* 96**,** 1378-82.

FALK, G., RICE, T., GOLDBLUM, J. & RICHTER, J. (1999) Jumbo biopsy forceps protocol still misses unsuspected cancer in Barrett's esophagus with high-grade dysplasia. *Gastrointestinal Endoscopy,* 49**,** 170-176.

FAN, X. & SNYDER, N. (2009) Prevalence of Barrett's Esophagus in Patients with or without GERD Symptoms: Role of Race, Age, and Gender. *Digestive Diseases and Sciences,* 54**,** 572-7.

FARRELL, J., TAUPIN, D., KOH, T., CHEN, D., ZHOU, C.-M., PODALSKY, D. & WANG, T. (2002) TFF2/SP-deficient mice show decreased gastric proliferation, increased acid secretion and increased susceptibility to NSAID injury. *Journal of Clinical Investigation,* 109**,** 193-204.

FERRAND, J., NOËL, D., LEHOURS, P., PROCHAZKOVA-CARLOTTI, M., CHAMBONNIER, L., MÉNARD, A., MÉGRAUD, F. & VARON, C. (2011)

Human Bone Marrow-Derived Stem Cells Acquire Epithelial Characteristics through Fusion with Gastrointestinal Epithelial Cells. *PLoS ONE,* 6**,** e19569.

FITZGERALD, R. (2006a) Molecular basis of Barrett's oesophagus and oesophageal adenocarcinoma. *Gut,* 55**,** 1810-1818.

FITZGERALD, R. (2006b) Molecular basis of Barrett's oesophagus and oesophageal adenocarcinoma. *Gut,* 55**,** 1810-18.

FOX, C., SAPINOSO, L., ZHANG, H., ZHANG, W., MCLEOD, H., PETRONI, G., MULLICK, T., MOSKALUK, C., FRIERSON, H., HAMPTON, G. & POWELL, S. (2005) Altered Expression of TFF-1 and CES-2 in Barrett's Esophagus and Associated Adenocarcinomas. *Neoplasia,* 7**,** 407-16.

FRANCHIMONT, D., COVAS, A., BRASSEUR, C., VAN LAETHEM, J.-L., EL-NAKADI, I. & DEVIERE, J. (2003) Newly Developed Barrett's Esophagus after Subtotal Esophagectomy. *Endoscopy,* 35**,** 850-853.

FRANKLIN, W., GAZDAR, A., HANEY, J., WISTUBA, I., LA ROSA, F., KENNEDY, T., RITCHEY, D. & MILLER, Y. (1997) Widely dispersed p53 mutation in respiratory epithelium. A novel mechanism for field carcinogenesis. *The Journal of Clinical Investigation,* 100**,** 2133-2137.

GALANDIUK, S., RODRIGUEZ-JUSTO, M., JEFFERY, R., NICHOLSON, A., CHENG, Y., OUKRIF, D., ELIA, G., LEEDHAM, S., MCDONALD, S., WRIGHT, N. & GRAHAM, T. (2012) Field Cancerization in the Intestinal Epithelium of Patients With Crohn's Ileocolitis. *Gastroenterology,* 142**,** 855-864.e8.

GALIPEAU, P., PREVO, L., SANCHEZ, C., LONGTON, G. & REID, B. (1999) Clonal Expansion and Loss of Heterozygosity at Chromosomes 9p and 17p in Premalignant Esophageal (Barrett's) Tissue. *Journal of the National Cancer Institute,* 91**,** 2087-2095.

GAMMON, M., SCHOENBERG, J., AHSAN, H., RISCH, H., VAUGHAN, T., CHOW, W., ROTTERDAM, H., WEST, A., DUBROW, R., STANFORD, J., MAYNE, S., FARROW, D., NIWA, S., BLOT, W. & FRAUMENI, J. (1997) Tobacco, alcohol, and socioeconomic status and adenocarcinomas of the esophagus and gastric cardia. *Journal of the National Cancer Institute,* 89**,** 1277-1284.

GATENBY, P., RAMUS, J., CAYGILL, C., FITZGERALD, R., CHARLETT, A., WINSLET, M. & WATSON, A. (2009) The influence of symptom type and

duration on the fate of the metaplastic columnar-lined Barrett's oesophagus. *Alimentary Pharmacology & Therapeutics,* 29**,** 1096-1105.

GERSON, L., SHETLER, K. & TRIADAFILOPOULOS, G. (2002) Prevalence of Barrett's esophagus in asymptomatic individuals. *Gastroenterology,* 123**,** 461-7.

GILLEN, P., KEELING, P., BYRNE, P., WEST, A. & HENNESY, T. (1988) Experimental columnar metaplasia in the canine oesophagus. *British Journal of Surgery,* 75**,** 113-115.

GLICKMAN, J., ORMSBY, A., GRAMICH, T., GOLDBLUM, J. & ODZE, R. (2005) Interinstitutional variability and effect of tissue fixative on the interpretation of a Barrett cytokeratin 7/20 immunoreactivity pattern in Barrett esophagus. *Human Pathology,* 36**,** 58-65.

GLICKMAN, J., WANG, H., DAS, K., GOYAL, R., SPECHLER, S., ANTONIOLI, D. & ODZE, R. (2001) Phenotype of Barrett's Esophagus and Intestinal Metaplasia of the Distal Esophagus and Gastroesophageal Junction. *The American Journal of Surgical Pathology,* 25**,** 87-94.

GRABOWSKI, P. & SCHINDLER, I. (2001) Neuroendocrine differentiation is a relevant prognostic factor in stage III-IV colorectal cancer. *European Journal of Gastroenterology and Hepatology,* 13**,** 405-411.

GRAY, M., DONNELLY, R. & KINGSNORTH, A. (1993) The role of smoking and alcohol in metaplasia and cancer risk in Barrett's columnar lined oesophagus. *Gut,* 34**,** 727-731.

GRIFFIN, M. & SWEENEY, E. (1987) The relationship of endocrine cells, dysplasia and carcinoembryonic antigen in Barrett's mucosa to adenocarcinoma of the oesophagus. *Histopathology,* 11**,** 53-62.

GRIFFIN SM & RAIMES SA (2006) *Oesophagogastric Surgery*, Elsevier Saunders.

GUILLEM, P. (2005) How to Make a Barrett Esophagus: Pathophysiology of Columnar Metaplasia of the Esophagus. *Digestive Diseases and Sciences,* 50**,** 415-424.

GUTSCHOW, C., VALLBÖHMER, D., STOLTE, M., OH, D., DANENBERG, K., DANENBERG, P., SCHNEIDER, P. & HÖLSCHER, A. (2008) Adenocarcinoma developing in de novo Barrett's mucosa in the remnant esophagus after esophagectomy: clinical and molecular assessment. *Diseases of the Esophagus,* 21**,** E6-E8.

HAFNER, C., TOLL, A., FERNANDEZ-CASADO, A., EARL, J., MARQUES, M., ACQUADRO, F., MENDEZ-PERTUZ, M., URIOSTE, M., MALATS, N., BURNS, J., KNOWLES, M., CIGUDOSA, J., HARTMANN, A., VOGT, T., LANDTHALER, M., PUJOL, R. & REAL, F. (2010) Multiple oncogenic mutations and clonal relationship in spatially distinct benign human epidermal tumors. *Proceedings of the National Academy of Sciences of the USA,* 107**,** 20780-20785.

HAGGITT, R., TRYZELAAR, J., ELLIS, F. & COLCHER, H. (1978) Adenocarcinoma complicating columnar epithelium-lined (Barrett's) esophagus. *American Journal of Clinical Pathology,* 70**,** 1-5.

HALL, P. & WATT, F. (1989) Stem cells: the generation and maintenance of cellular diversity. *Development,* 106**,** 619-33.

HAMEETEMAN, W., TYTGAT, G. N., HOUTHOFF, H. J. & TWEEL, J. G. V. D. (1989) Barrett's esophagus: Development of dysplasia and adenocarcinoma. *Gastroenterology,* 96**,** 1249-1256.

HAMILTON, K., CHIAPPORI, A., OLSON, S., SAWYERS, J., JOHNSON, D. & WASHINGTON, K. (2000) Prevalence and Prognostic Significance of Neuroendocrine Cells in Esophageal Adenocarcinoma. *Modern Pathology,* 13**,** 475-481.

HAMILTON, S. & YARDLEY, J. (1977) Regeneration of Cardiac Type Mucosa and Acquisition of Barrett Mucosa after Esophagogastrostomy. *Gastroenterology,* 72**,** 669-675.

HAMZA, A., ABDELHAY, S., SHERIF, H., HASAN, T., SOLIMAN, H., KABESH, A., BASSIOUNY, I. & BAHNASSY, A. (2003) Caustic Esophageal Strictures in Children: 30 Years Experience. *Journal of Paediatric Surgery,* 38**,** 828-833.

HANBY, A., JANKOWSKI, J., ELIA, G., POULSOM, R. & WRIGHT, N. (1994) Expression of the trefoil peptides pS2 and human spasmolytic polypeptide (hSP) in Barrett's metaplasia and the native oesophageal epithelium: Delineation of epithelial phenotype. *Journal of Pathology,* 173**,** 213-219.

HANBY, A., POULSOM, R., ELIA, G., SINGH, S., LONGCROFT, J. & WRIGHT, N. (1993a) The expression of the trefoil peptides pS2 and human spasmolytic polypeptide (hSP) in 'gastric metaplasia' of the proximal

duodenum: implications for th enature of 'gastric metaplasia'. *Journal of Pathology,* 169**,** 355-360.

HANBY, A., POULSOM, R., SINGH, S., ELIA, G., JEFFREY, R. & WRIGHT, N. (1993b) Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. *Gastroenterology,* 105**,** 1110-1116.

HARRISON R, PERRY I, HADDADIN W, MCDONALD S, BRYAN R, ABRAMS K, SAMPLINER R, TALLEY NJ, MOAYYEDI P & JA., J. (2007) Detection of intestinal metaplasia in Barrett's esophagus: an observational comparator study suggests the need for a minimum of eight biopsies. *American Journal of Gastroenterology,* 102**,** 1154-61.

HAUSER, F., POULSOM, R., CHINERY, R., ROGERS, L., HANBY, A., WRIGHT, N. & HOFFMAN, W. (1993) hP1.B, a human P-domain peptide homolougous with rat intestinal trefoil factor, is expressed also in the ulcer-associated cell lineage and the uterus. *Proceedings of the National Academy of Sciences of the USA,* 90**,** 6961-5.

HAVU, N. (1986) Enterochromaffin-like cell carcinoids of gastric mucosa in rats after life-long inhibition of gastric secretion. *Digestion,* 35(Suppl.)**,** 42-55.

HAYWARD, J. (1961) The lower end of the oesophagus. *Thorax,* 16**,** 36-41.

HEITMILLER, R., REDMOND, M. & HAMILTON, S. (1996) Barrett's esophagus with high grade dysplasia. An indication for prophylactic esophagectomy. *Annals of Surgery,* 224**,** 66-71.

HENRY, J., BENNETT, M., PIGGOTT, N., LEVETT, D., MAY, F. & WESTLEY, B. (1991) Expression of the pNR-2/pS2 protein in diverse human epithelial tumours. *British Journal of Cancer,* 64**,** 677-82.

HIRSCHOWITZ, B. (1996) Gastric acid and pepsin secretion in patients with Barrett's esophagus and appropriate controls. *Digestive Diseases and Sciences,* 41**,** 1384-1391.

HU, Y., JONES, C., GELLERSEN, O., WILLIAMS, V., WATSON, T. & PETERS, J. (2007) Pathogenesis of barrett esophagus: Deoxycholic acid up-regulates goblet-specific gene muc2 in concert with cdx2 in human esophageal cells. *Archives of Surgery,* 142**,** 540-545.

HVID-JENSEN, F., PEDERSEN, L., DREWES, A., SØRENSEN, H. & FUNCH-JENSEN, P. (2011) Incidence of Adenocarcinoma among Patients with Barrett's Esophagus. *New England Journal of Medicine,* 365**,** 1375-83.

JANKOWSKI, J. & BARR, H. (1996) Improving surveillance for Barrett's oesophagus: AspECT and BOSS trials provide an evidence base. *British Medical Journal,* 332**,** 1512.

JANKOWSKI, J., HARRISON, R., PERRY, I., BALKWILL , F. & TSELEPIS, C. (2000) Barrett's metaplasia. *The Lancet,* 356**,** 2079-2085.

JANKOWSKI, J., MCMENEMIN, R., HOPWOOD, D., PENSTON, J. & WORMSLEY, K. (1991) Abnormal expression of growth regulatory factors in Barrett's oesophagus. *Clinical Sciences,* 81**,** 663-8.

JANKOWSKI, J., WRIGHT, N., MELTZER, S., TRIADAFILOPOULOS, G., GEBOES, K., CASSON, A., KERR, D. & YOUNG, L. (1999) Molecular Evolution of the Metaplasia-Dysplasia-Adenocarcinoma Sequence in the Esophagus. *American Journal of Pathology,* 154**,** 965-973.

JASKIEWICZ, K., LOUW, J. & ANICHKOV, N. (1994) Barrett's oesophagus, mucin composition, neuroendocrine cells, p53 protein, celular proliferation and differentiation. *Anticancer Research* 14**,** 1907-12.

JOHANSSON, J., HAKANSSON, H.-O., MELLBLOM, L., KEMPAS, A., JOHANSSON, K.-E., GRANATH, F. & NYREN, O. (2007) Risk factors for Barrett's oesophagus: A population-based approach. *Scandinavian Journal of Gastroenterology,* 42**,** 148 - 156.

JOHANSSON, J., JOHNSSON, F., GROSHEN, S. & WALTHER, B. (1999) Pharyngeal reflux after gastric pull-up esophagectomy with neck and chest anastomoses. *Journal of thoracic and cardiovascular surgery,* 118**,** 1078-1083.

JORGENSEN, K., THIM, L. & JACOBSEN, H. (1982) Pancreatic spasmolytis polypeptide (PSP): 1. Preparation and initial chemical characterization of a new polypeptide from porcine pancreas. *Regulatory peptides,* 3**,** 207-19.

JOVANOVIC, I., TZARDI, M., MOUZAS, I., MICEV, M., PESCO, P., MILOSAVLJEVIC, T., ZOIS, M., SGANZOS, M., DELIDES, G. & KANAVAROS, P. (2002) Changing pattern of cytokeratin 7 and 20 expression from normal epithelium to intestinal metaplasia of the gastric mucosa and gastroesophageal junction. *Histology and Histopathology,* 17**,** 445-454.

KADRI SR, LAO-SIRIEIX P, O'DONOVAN M, DEBIRAM I, DAS M, BLAZEBY JM, EMERY J, BOUSSIOUTAS A, MORRIS H, WALTER FM,

PHAROAH P, HARDWICK RH & RC, F. (2010) Acceptability and accuracy of a non-endoscopic screening test for Barrett's oesophagus in primary care: cohort study. *British Medical Journal,* 341**,** c4372 (online first).

KIM, D., KIM, J., CHO, J., BAEK, S., KAKAR, S., KIM, G., SLEISENGER, M. & KIM, Y. (2005) Expression of mucin core proteins, trefoil factors, APC and p21 in subsets of colorectal polyps and cancers suggests a distinct pathway of pathogenesis of mucinous carcinoma of the colorectum. *International Journal of Oncology,* 27**,** 957-64.

KIM, Y. & HO, S. (2010) Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress. *Current Gastroenterology Reports,* 12**,** 319-30.

KJELLEV, S. (2009) The trefoil factor family – small peptides with multiple functionalities. *Cellular and Molecular Life Sciences,* 66**,** 1350-1369.

KOPPERT, L., WIJNHOVEN, B., TILANUS, H., STIJNEN, T., VAN DEKKEN, H. & DINJENS, W. (2004) Neuroendocrine Cells in Barrett's Mucosa and Adenocarcinomas of the Gastroesophageal Junction. *International Journal of Surgical Pathology,* 12**,** 117-125.

KOUZNETSOVA, I., KALINSKI, T., PEITZ, U., MONKEMULLER, K., KALBACHER, H., VIETH, M., MEYER, F., ROESSNER, A., MALFERTHEINER, P., LIPPERT, H. & HOFFMAN, W. (2007) Localization of TFF3 peptide in human esophageal submucosal glands and gastric cardia: differentiation of two types of gastric pit cells along the rostro-caudal axis. *Cell Tissue Research,* 328**,** 365-374.

KULIG, M., NOCON, M., VIETH, M., LEODOLTER, A., JASPERSEN, D., LABENZ, J., MEYER-SABELLEKE, W., STOLTEF, M., LINDG, T., MALFERTHEINERB, P. & WILLICH, S. (2004) Risk factors of gastroesophageal reflux disease: methodology and first epidemiological results of the ProGERD study. *Journal of clinical Epidemiology,* 57**,** 580-9.

LABOUVIE, C., MACHADO, J., CARNEIRO, F., SARBIA, M., VEITH, M., PORSCHEN, R., SEITZ, G. & BLIN, N. (1999) Differential expression of mucins and trefoil peptides in native epithelium, Barrett's metaplasia and squamous cell carcinoma of the oesophagus. *Journal of cancer research and clinical oncology,* 125**,** 71-76.

LAGERGREN, J., BERGSTROM, R., LINDGREN, A. & NYREN, O. (1999) Symptomatic Gastroesophageal Reflux as a Risk Factor for Esophageal Adenocarcinoma. *New England Journal of Medicine,* 340**,** 825-831.

LAGERGREN, J., BERGSTROM, R., LINDGREN, A. & NYREN, O. (2000) The role of tobacco, snuff, and alcohol use in the aetiology of cancer of the oesophagus and gastric cardia. *International Journal of Cancer,* 85**,** 340-346.

LAINE, L., AHNEN, D., MCCLAIN, C., SOLCIA, E. & WALSH, J. (2000) Review article: potential gastrointestinal effects of long-term acid suppression with proton pump inhibitors. *Alimentary Pharmacology & Therapeutics,* 14**,** 651-68.

LAMBERTS, R., CREUTZFELDT, W., STRÜBER, H., BRUNNER, G. & SOLCIA, E. (1993) Long-term omeprazole therapy in peptic ulcer disease: gastrin, endocrine cell growth, and gastritis. *Gastroenterology,* 104**,** 1356-70.

LAU-SIRIEIX, P., BOUSSIOUTAS, A., KADRI, S., O'DONOVAN, M., DEBIRAM, I., DAS, M., HARIHAR, L. & FITZGERALD, R. (2009) Non-endoscopic screening biomarkers for Barrett's oesophagus: from microarray analysis to the clinic. *Gut,* 58**,** 1451-1459.

LEEDHAM, S., GRAHAM, T., OUKRIF, D., MCDONALD, S., RODRIGUEZ-JUSTO, M., HARRISON, R., SHEPHERD, N., NOVELLI, M., JANKOWSKI, J. & WRIGHT, N. (2009) Clonality, Founder Mutations, and Field Cancerization in Human Ulcerative Colitis Associated Neoplasia. *Gastroenterology,* 136**,** 542-550.e6.

LEEDHAM, S. J., PRESTON, S. L., MCDONALD, S. A. C., ELIA, G., BHANDARI, P., POLLER, D., HARRISON, R., NOVELLI, M. R., JANKOWSKI, J. A. & WRIGHT, N. A. (2008) Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. *Gut,* 57**,** 1041-1048.

LEFEBVRE, O., CHENARD, M., MASSON, R., LINARES, J., DIERICH, A., LEMEUR, M., WENDLING, C., TOMASETTO, C., CHAMBON, P. & RIO, M. (1996) Gastric mucosa abnormalities and tumourigenesis in mice lacking the pS2 trefoil protein. *Science,* 275**,** 259-62.

LIEBERMAN, D., OEHIKE, M. & HELFAND, M. (1997) Risk factors for Barrett's esophagus in community based practice. *American Journal of Gastroenterology,* 92**,** 1293-1297.

LILLEMOE, K., JOHNSON, L. & HARMON, J. (1982) Role of the components of the gastroduodenal contents in experimental acid esophagitis. *Surgery,* 92**,** 276-282.

LINDAHL, H., RINTALA, R., SARIOLA, H. & LOUHIMO, I. (1990) Cervical Barrett's Esophagus: A Common Complication of Gastric Tube Reconstruction. *Journal of Pediatric Surgery,* 25**,** 446-448.

LIU, T., ZHANG, X., SO, C.-K., WANG, S., WANG, P., YAN, L., MYERS, R., CHEN, Z., PATTERSON, A., YANG, C. & CHEN, X. (2006a) Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis,* 28**,** 488-496.

LIU, T., ZHANG, X., SO, C., WANG, S., WANG, P., YAN, L., MYERS, R., CHEN, Z., PATTERSON, A., YANG, C. & CHEN, X. (2006b) Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis,* 28**,** 488-496.

LOCKE, G. R., TALLEY, N. J., FETT, S. L., ZINSMEISTER, A. R. & MELTONIII, L. J. (1999) Risk factors associated with symptoms of gastroesophageal reflux. *The American Journal of Medicine,* 106**,** 642-649.

LONGMAN, R., DOUTHWAITE, J., SYLVESTER, P., POULSOM, R., CORFIELD, A., THOMAS, M. & WRIGHT, N. (2000) Coordinated localisation of mucins and trefoil peptides in the ulcer associated cell lineage and the gastrointestinal mucosa. *Gut,* 47**,** 792-800.

LORD, R., WICKRAMASINGHE, K., JOHANSON, J., DEMEESTER, S., BRABENDER, J. & DEMEESTER, T. (2004) Cardiac mucosa in the remnant esophagus after esophagectomy is an acquired epithelium with Barrett's-like features. *Surgery,* 136**,** 633-40.

LUKETICH, J., SCHAUER, P., CHRISTIE, N., WEIGEL, T., RAJA, S., FERNANDO, H., KEENAN, R. & NGUYEN, N. (2000) Minimally Invasive Esophagectomy. *The Annals of Thoracic Surgery,* 70**,** 906-11.

MACHADO, J., CARNEIRO, F., BLIN, N. & SOBRINHO-SIMOES, M. (1996) Pattern of pS2 protein expression in premalignant and malignant lesions of gastric mucosa. *European Journal of Cancer prevention,* 5**,** 169-79.

MADSEN, J., NIELSEN, O., TORNOE, I., THIM, L. & HOLMSKOV, U. (2007) Tissue Localization of Human Trefoil Factors 1, 2, and 3. *Journal of Histochemistry and Cytochemistry,* 55**,** 505-513.

MALEY, C., GALIPEAU, P., LI, X., SANCHEZ, C., PAULSON, T. & REID, B. (2004) Selectively Advantageous Mutations and Hitchhikers in Neoplasms. *Journal of the National Cancer Institute,* 64**,** 3414-3427.

MASHIMO, H., WU, D., PODALSKY, D. & FISHMAN, M. (1996) Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science,* 274**,** 262-265.

MASIAKOWSKI, P., BREATHNACH, R., BLOCH, J., GANNON, F., KRUST, A. & CHAMBON, P. (1982) Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Research,* 10**,** 7895-7903.

MATTSSON, H., HAVU, N., BRAUTIGAM, J., CARLSSON, K., LUNDELL, L. & CARLSSON, E. (1991) Partial gastric corpectomy results in hypergasrinemia and development of gastric enterochromaffin-like cell carcinoids in the rat. *Gastroenterology,* 100**,** 311-19.

MCKEOWN, K. (1976) Total three-stage oesophagectomy for cancer of the oesophagus. *British Journal of Surgery,* 63**,** 259-262.

MEDICAL RESEARCH COUNCIL OESOPHAGEAL CANCER WORKING GROUP (2002) Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. *The Lancet,* 359**,** 1727-1733.

MEEK, D. (2009) Tumour suppression by p53: a role for the DNA damage response? *Nature reviews cancer,* 9**,** 714-23.

MELO, L., KRUEL, KLIEMANN, CAVAZZOLA, BOENO, R., SILBER & GROSSI (1999) Influence of surgically induced gastric and gastroduodenal content reflux on esophageal carcinogenesis – experimental model in Wistar female rats. *Diseases of the Esophagus,* 12**,** 106-115.

MEYER, W., VOLLMAR, F. & BAR, W. (1979a) Barrett-esophagus following total gastrectomy. A contribution to its pathogenesis. *Endoscopy,* 11**,** 121-126.

MEYER, W., VOLLMAR, F. & BAR, W. (1979b) Barrett-esophagus following total gastrectomy. A contribution to it's pathogenesis. *Endoscopy,* 11**,** 121-6.

MOHAMMED, I., STREUTKER, C. & RIDDELL, R. (2002) Utilization of Cytokeratins 7 and 20 Does Not Differentiate between Barrett's Esophagus and Gastric Cardiac Metaplasia. *Modern Pathology,* 15**,** 611-616.

MORSON, B. & BELCHER, J. (1952) Adenocarcinoma of the oesophagus and ectopic gastric mucosa. *British Journal of Cancer,* 6**,** 127-130.

MULHOLLAND, M., REID, B., LEVINE, D. & RUBIN, C. (1989) Elevated Gastric Acid Secretion in Patients with Barrett's Metaplastic Epithelium. *Digestive Diseases and Sciences,* 34**,** 1329-1335.

MULLER, W. & BORCHARD, F. (1993) pS2 protein in gastric carcinoma and normal gastric mucosa: association with clinicopahological parameters and patient survival. *Journal of Pathology,* 171**,** 263-9.

NAEF, A., SAVARY, M. & OZZELLO, L. (1975) Columnar-lined lower esophagus: an acquired lesion with malignant predisposition. Report on 140 cases of Barrett's esophagus with 12 adenocarcinomas. *The Journal of Thoracic and Cardiovascular Surgery,* 70**,** 826-35.

NICHOLSON, A., GRAHAM, T., SIMPSON, A., HUMPHRIES, A., BURCH, N., RODRIGUEZ-JUSTO, M., NOVELLI, M., HARRISON, R., WRIGHT, N., MCDONALD, S. & JANKOWSKI, J. (2012) Barrett's metaplasia glands are clonal, contain multiple stem cells and share a common squamous progenitor. *Gut,* 61**,** 1380-1389.

NISHIMURA, K., TANAKA, T., TANAKA, K., MATONO, S., MURATA, K., SHIROUZU, K. & FUJITA, H. (2010) Reflux esophagitis and columnar-lined esophagus after cervical esophagogastrostomy (following esophagectomy). *Diseases of the Esophagus,* 23**,** 94-99.

NOSHO, K., KURE, S., IRAHARA, N., SHIMA, K., BABA, Y., SPIEGELMAN, D., MEYERHARDT, J., GIOVANNUCCI, E., FUCHS, C. & OGINO, S. (2009) A Prospective Cohort Study Shows Unique Epigenetic, Genetic, and Prognostic Features of Synchronous Colorectal Cancers. *Gastroenterology,* 137**,** 1609-1620.e3.

O'RIORDAN, J., TUCKER, O., BYRNE, P., MCDONALD, G., RAVI, N., KEELING, P. & REYNOLDS, J. (2004) Factors Influencing The

Development of Barrett's Epithelium in The Esophageal Remnant Postesophagectomy. *American Journal of Gastroenterology,* 99**,** 205-211.

OBERG, S., JOHANSSON, J., WENNER, J. & WALTHER, B. (2002) Metaplastic Columnar Mucosa in the Cervical Esophagus After Esophagectomy. *Annals of Surgery,* 235**,** 338-345.

OH, D. & DEMEESTER, S. (2010) Pathophysiology and treatment of Barrett's esophagus. *World Journal of Gastroenterology,* 16**,** 3762-72.

ORMSBY, A., GOLDBLUM, J., RICE, T., RICHTER, J., FALK, G., VAEZI, M. & GRAMLICH, T. (1999) Cytokeratin subsets can reliably distinguish Barrett's esophagus from intestinal metaplasia of the stomach. *Human Pathology,* 30**,** 288-294.

ORMSBY, A., KILGORE, S., GOLDBLUM, J., RICHTER, J., RICE, T. & GRAMLICH, T. (2000) The Location and Frequency of Intestinal Metaplasia at the Esophagogastric Junction in 223 Consecutive Autopsies: Implications for Patient Treatment and Preventive Strategies in Barrett's Esophagus. *Mod Pathol,* 13**,** 614-620.

PAULL, A., TRIER, J. S., DALTON, M. D., CAMP, R. C., LOEB, P. & GOYAL, R. K. (1976) The histologic spectrum of Barrett's esophagus. *The New England Journal of Medicine,* 295**,** 476-480.

PAULSON, T., XU, L., SANCHEZ, C., BLOUNT, P., AYUB, K., ODZE, R. & REID, B. (2006) Neosquamous Epithelium Does Not Typically Arise from Barrett's Epithelium. *Clinical Cancer Research,* 12**,** 1701-1706.

PEITZ, U., KOUZNETSOVA, I., WEX, T., GEBERT, I., VIETH, M., ROESSNER, A., HOFFMANN, W. & MALFERTHEINER, P. (2004a) TFF3 expression at the esophagogastric junction is increased in gastro-esophageal reflux disease (GERD). *Peptides,* 25**,** 771-777.

PEITZ, U., VIETH, M., PROSS, M., LEODOLTER, A. & MALFERTHEINER, P. (2004b) Cardia-type metaplasia arising in the remnant esophagus after cardia resection. *Gastrointestinal Endoscopy,* 59**,** 810-817.

PERA, M., CARDESA, A., BOMBI, J., ERNST, H., PERA, C. & MOHR, U. (1989) Influence of Esophagojejunostomy on the Induction of Adenocarcinoma of the Distal Esophagus in Sprague-Dawley Rats by Subcutaneous Injection of 2,6-Dimethylnitrosomorpholine. *Cancer Research,* 49**,** 6803-6808.

PLAYFORD, R., MARCHBANK, T., CHINERY, R., EVISON, R., PIGNATELLI, M., BOULTON, R., THIM, L. & HANBY, A. (1995) Human spasmolytic polypeptided is a cytoprotective agent taht stimulates cell migration. *Gastroenterology,* 108**,** 108-16.

PODALSKY, D., LYNCH-DEVANEY, K., STOW, J., OATES, P., MURGUE, B., DEBEAUMONT, M., SANDS, B. & MAHIDA, Y. (1993) Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. *Journal of biological chemistry,* 268**,** 6694-702.

POHL, H. & WELCH, H. G. (2005) The Role of Overdiagnosis and Reclassification in the Marked Increase of Esophageal Adenocarcinoma Incidence. *Journal of the National Cancer Institute,* 97**,** 142-146.

PRACH, A., MACDONALD, T., HOPWOOD, D. & JOHNSTON, D. (1997a) Increasing incidence of Barrett's oesophagus: education, enthusiasm, or epidemiology? *The Lancet,* 350**,** 933.

PRACH, A., MACDONALD, T., HOPWOOD, D. & JOHNSTONE, D. (1997b) Increasing incidence of Barrett's oesophagus: education, enthusiasm or epidemiology *The Lancet,* 350**,** 933.

PRATEEK, S., JOHN, D., DAVID, A., JACQUES, J. G. H. M. B., LIEBWIN, G., YOSHIO, H., JANUSZ, A. J., OLA, J., LARS, L., GUIDO, N. J. T. & MICHAEL, V. (2006) The Development and Validation of an Endoscopic Grading System for Barrett's Esophagus: The Prague C & M Criteria. *Gastroenterology,* 131**,** 1392-1399.

REED, M., TOLIS, G. J., EDIL, B., ALLAN, J., DONAHUE, D., GAISSERT, H., MONCURE, A., WAIN, J., WRIGHT, C. & MATHISEN, D. (2005) Surgical treatment of esophageal high-grade dysplasia. *Annals of Thoracic Surgery,* 75**,** 1110-1115.

REGALO, G., WRIGHT, N. & MACHADO, J. (2005) Trefoil factors: from ulceration to neoplasia. *Cellular and Molecular Life Sciences,* 62**,** 2910-2915.

REINHARDT, H. & SCHUMACHER, B. (2012) The p53 network: cellular and systemic DNA damage responses in aging and cancer. *Trends in Genetics,* 28**,** 128-136.

REIS, C., DAVID, L., CORREA, P., CARNEIRO, F., DE BOLAS, C., GARCIA, E., MANDEL, U., CLAUSEN, H. & SOBRINHO-SIMOES, M. (1999)

Intestinal Metaplasia of Human Stomach Displays Distinct Patterns of Mucin (MUC1, MUC2, MUC5AC, and MUC6) Expression. *Cancer Research,* 59**,** 1003-1007.

RIDDELL, R., GOLDMAN, H., RANSOHOFF, D., APPELMAN, H., FENOGLIO, C., HAGGITT, R., HREN, C., CORREA, P., HAMILTON, S., MORSON, B., SOMMERS, S. & YARDLEY, J. (1983) Dysplasia in inflamatory bowel disease. Standardised classification with provisional clinical application. *Human Pathology,* 14**,** 931-966.

RIO, M., BELLOCQ, J., DANIEL, J., TOMASETTO, C., LATHE, R., CHENARD, M., BATZENSCHLAGER, A. & CHAMBON, P. (1988) Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. *Science,* 241**,** 705-8.

ROGERS, L. & MURPHY, R. (1979) Gastric carcinoid and gastric carcinoma. *American Journal of Surgical Pathology,* 3.

RONKAINEN, J., ARO, P., STORSKRUBB, T., JOHANSSON, S., LIND, T., BOLLING-STERNEVALD, E., VIETH, M., STOLTE, M., TALLEY, N. & AGREUS, L. (2005) Prevalence of Barrett's Esophagus in the General Population: An Endoscopic Study. *Gastroenterology,* 129**,** 1825-1831.

RUBENSTEIN, J., SAINI, S., KUHN, L., MCMAHON, L., SHARMA, P., PARDI, D. & SCHOENFELD, P. (2008) Influence of Malpractice History on the Practice of Screening and Surveillance for Barrett's Esophagus. *Am J Gastroenterol,* 103**,** 842-849.

RUDOLPH RE, VAUGHAN TL, STORER BE, HAGGITT RC, RABINOVITCH PS, LEVINE DS & BJ., R. (2000) Effect of segment length on risk for neoplastic progression in patients with Barrett esophagus. *Annals of Internal Medicine,* 132**,** 612-620.

SANDULEANU, S., STRIDSBERG, M., JONKERS, D., HAMEETEMAN, W., BIEMOND, I., LUNDQVIST, G., LAMERS, C. & STOCKBRÜGGER, R. (1999) Serum gastrin and chromogranin A during medium- and long-term acid suppressive therapy: a case-control study. *Alimentary Pharmacology & Therapeutics,* 13**,** 145-153.

SAROSI, G., BROWN, G., JAISWAL, K., FEAGINS, L., LEE, E., CROOK, T., SOUZA, R., ZOU, Y., SHAY, J. & SPECHLER, S. (2008) Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in

a rat model of Barrett's esophagus. *Diseases of the Esophagus,* 21**,** 43-50.

SCHNEIDER, P., STOELTZING, O., ROTH, J., HOELSCHER, A., WEGERER, S., MIZUMOTO, S., BECKER, K., DITTLER, H., FINK, U. & SIEWERT, J. (2000) p53 Mutational Status Improves Estimation of Prognosis in Patients with Curatively Resected Adenocarcinoma in Barrett's Esophagus. *Clinical Cancer Research,* 6**,** 3153-3158.

SCHNELL, T., SONTAG, S. & CHEJFEC, G. (1992) Adenocarcinomas arising in tongues or short segments of Barrett's esophagus. *Dig Dis Sci,* 37**,** 137-143.

SCHNELL, T., SONTAG, S., CHEJFEC, G., ARANHA, G., METZ, A., O'CONNELL, S., SEIDEL, U. & SONNENBERG, A. (2001) Long-term nonsurgical management of Barrett's esophagus with high-grade dysplasia. *Gastroenterology,* 120**,** 1607-19.

SEERY, J. (2002) Stem cells of the oesophageal epithelium. *Journal of Cell Science,* 115**,** 1783-89.

SEERY, J. & WATT, F. (2000) Asymmetric stem-cell divisions define the architecture of the human oesophageal epithelium. *Current Biology,* 10**,** 1447-1450.

SHAHEEN, N., CROSBY, M., BOZYMSKI, E. & SANDLER, R. (2000) Is there publication bias in the reporting of cancer risk in Barrett's esophagus? *Gastroenterology,* 119**,** 333-338.

SHEARER, C., GOING, J., NEILSON, L., MACKAY, C. & STUART, R. (2005) Cytokeratin 7 and 20 expression in intestinal metaplasia of the distal oesophagus: relationship to gastro-oesophageal reflux disease. *Histopathology,* 47**,** 268-275.

SHIBUYA, S., FUKUDO, S., SHINEHA, R., MIYAZAKI, S., MIYATA, G., SUGAWARA, K., MORI, T., TANABE, S., TONOTSUKA, N. & SATOMI, S. (2003) High Incidence of Reflux Esophagitis Observed by Routine Endoscopic Examination after Gastric Pull-up Esophagectomy. *World Journal of Surgery,* 27**,** 580-583.

SKACEL, M., PETRAS, R., GRAMLICH, T., SIGEL, J., RICHTER, J. & GOLDBLUM, J. (2000) The diagnosis of low-grade dysplasia in Barrett's esophagus and its implications for disease progression. *American Journal of Gastroenterology,* 95**,** 3383-3387.

SLAUGHTER, D., SOUTHWICK, H. & SMEJKAL, W. (1953) Field canceriazation in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer,* 6**,** 963-968.

SMITH, D. & HAGGITT, R. (1984) The prevalence and prognostic significance of argyrophil cells in colorectal carcinomas. *American Journal of Surgical Pathology,* 8**,** 123-128.

SOMMERER, F., VIETH, M., MARKWARTH, A., RÖHRICH, K., VOMSCHLOSS, S., MAY, A., ELL, C., STOLTE, M., HENGGE, U., WITTEKIND, C. & TANNAPFEL, A. (2004) Mutations of BRAF and KRAS2 in the development of Barrett's adenocarcinoma. *Oncogene,* 23**,** 554-8.

SPECHLER, S., SHARMA, P., SOUZA, R., INADOMI, J. & SHAHEEN, N. (2011) American Gastroenterological Association Technical Review on the Management of Barrett's Esophagus. *Gastroenterology,* 140**,** e18-e13.

SPITZ, L., KIELY, E. & PIERRO, A. (2004) Gastric Transposition in Children - A 21-year Experience. *Journal of Paediatric Surgery,* 39**,** 276-281.

SUEMORI, S., LYNCH-DEVANEY, K. & PODALSKY, D. (1991) Identification and characterization of rat intestinal trefoil factor: tissue adn cell-specific member of the trefoil protein family. *Proceedings of the National Academy of Sciences of the USA,* 88**,** 17-21.

TAKUBO, K., AIDA, J., NAOMOTO, Y., SAWABE, M., ARAI, T., SHIRAISHI, H., MATSUURA, M., ELL, C., MAY, A., PECH, O., STOLTE, M. & VIETH, M. (2009) Cardiac rather than intestinal-type background in endoscopic resection specimens of minute Barrett adenocarcinoma. *Human pathology,* 40**,** 65-74.

THEISEN, J., NIGRO, J., DEMEESTER, T., PETERS, J., GASTAL, O., HAYGEN, J., HASHEMI, M. & BREMNER, C. (2004) Chronology of the Barrett's metaplasia-dysplasia-carcinoma sequence. *Diseases of the Esophagus,* 17**,** 67-70.

THEISEN, J., STEIN, H., DITTLER, H., FEITH, M., MOEBIUS, C., KAUER, W., WERNER, M. & SIEWERT, J. (2002) Preoperative chemotherapy unmasks underlying Barrett's mucosa in patients with adenocarcinoma of the distal esophagus. *Surgical Endoscopy,* 16**,** 671-673.

THIM, L. (1989) A new family of growth factor-like peptides [`]Trefoil' disulphide loop structures as a common feature in breast cancer associated peptide (pS2), pancreatic spasmolytic polypeptide (PSP), and frog skin peptides (spasmolysins). *FEBS Letters,* 250**,** 85-90.

THOMAS, T., ABRAMS, K., DE CAESTECKER, J. & ROBINSON, R. (2007) Meta analysis: cancer risk in Barrett's oesophagus. *Alimentary Pharmacology and Therapeutics,* 26**,** 1465-77.

TILESTON, W. (1906) Peptic ulcer of the oesophagus. *American Journal of Medical Science,* 132**,** 240-265.

TSIOURIS, A., HAMMOND, Z. & VELANOVICH, V. (2011) Barrett's Esophagus After Resection of the Gastroesophageal Junction: Effects of Concomitant Fundoplication. *World Journal of Surgery,* 35**,** 1867-72.

UNDERWOOD, J. (1998) *General and Systematic Pathology*, Churchill Livingstone.

VAEZI, M. & RICHTER, J. (1996) Role of Acid and Duodenogastroesophageal Reflux in Gastroesophageal Reflux Disease. *Gastroenterology,* 111**,** 1192-1199.

VAHABZADEH, B., SEETHARAM, A., COOK, M., WANI, S., RASTOGI, A., BANSAL, A., EARLY, D. & SHARMA, P. Validation of the Prague C & M criteria for the endoscopic grading of Barrett's esophagus by gastroenterology trainees: a multicenter study. *Gastrointestinal endoscopy,* 75**,** 236-241.

VAN DE BOVENKAMP, J., KORTELAND-VAN MALE, A., WARSON, C., BULLER, H., EINERHAND, A., ECTORS, N. & DEKKER, J. (2003) Gastric-type mucin and TFF-peptide expression in Barrett's oesophagus is disturbed during increased expression of MUC2. *Histopathology,* 42**,** 555-565.

VAN LANSCHOT, J., HULSCHER, J., BUSKENS, C., TILANUS, H., TEN KATE, F. & OBERTOP, H. (2001) Hospital volume and hospital mortality for esophagectomy. *Cancer,* 91**,** 1574-1578.

VAN SOEST, E. M., DIELEMAN, J. P., SIERSEMA, P. D., STURKENBOOM, M. C. J. M. & KUIPERS, E. J. (2005) Increasing incidence of Barrett's oesophagus in the general population. *Gut,* 54**,** 1062-1066.

VOUTILAINEN, M., JUHOLA, M., PITKANEN, R., FARKKILA, M. & SIPPONEN, P. (2002) Immunohistochemical study of neuroendocrine cells at the gastric cardia mucosa. *Journal of clinical Pathology,* 55**,** 767-69.

WANG, D. & SOUZA, R. (2011) Biology of Barrett's Esophagus and Esophageal Adenocarcinoma. *Gastrointestinal Endoscopy Clinics of North America,* 21**,** 25-38.

WANG, K. & SAMPLINER, R. E. (2008) Updated Guidelines 2008 for the Diagnosis, Surveillance and Therapy of Barrett's Esophagus. *American Journal of Gastroenterology,* 103**,** 788-797.

WANG, K., YANG, Q., CLEARY, K., SWISHER, S., CORREA, A., KOMAKI, R., AJANI, J., RASHID, A., HAMILTON, S. & WU, T.-T. (2006) The Significance of Neuroendocrine Differentiation in Adenocarcinoma of the Esophagus and Esophagogastric Junction after Preoperative Chemoradiation. *Cancer,* 107**,** 1467-74.

WANG, X., OUYANG, H., YAMAMOTO, Y., KUMAR, P., WEI, T., DAGHER, R., VINCENT, M., LU, X., BELLIZZI, A., HO, K., CRUM, C., XIAN, W. & MCKEON, F. (2011) Residual Embryonic Cells as Precursors of a Barrett's-like Metaplasia. *Cell,* 145**,** 1023-1035.

WARSON, C., VAN DE BOVENKAMP, J., KORTELAND-VAN MALE, A., BULLER, H., EINERHAND, A., ECTORS, N. & DEKKER, J. (2002) Barrett's Esophagus is Characterized by Expression of Gastric-type Mucins (MUC5AC, MUC6) and TFF Peptides (TFF1 and TFF2), but the Risk of Carcinoma Development my be Indicated by the Intestinal-type Mucin, MUC2. *Human Pathology,* 33**,** 660-668.

WATSON, A., HEADING, R. & SHEPHERD, N. (Eds.) (2005) *Guidelines for the diagnosis and management of Barrett's columnar-lined oesophagus*, British Society of Gastroenterology.

WESTHOFF, B., WESTON, A., CHERIAN, R. & SHARMA, P. (2004) Development of Barrett's Esophagus Six Months after Total Gastrectomy. *American Journal of Gastroenterology,* 99**,** 2271-2277.

WESTON, A., BANERJEE, S., SHARMA, P., TRAN, T., RICHARDS, R. & CHERIAN, R. (2001) p53 protein overexpression in low grade dysplasia (LGD) in Barrett's esophagus: immunohistochemical marker predictive of progression. *American Journal of Gastroenterology,* 96**,** 1355-1362.

WESTON, A., SHARMA, P., TOPALOVSKI, M., RICHARDS, R., CHERIAN, R. & DIXON, A. (2000) Long-term follow-up of Barrett's high-grade dysplasia. *American Journal of Gastroenterology,* 95**,** 1888-1893.

WESTON AP, SHARMA P, MATHUR S, BANERJEE S, JAFRI AK, CHERIAN R, MCGREGOR D, HASSANEIN RS & M., H. (2004) Risk stratification of Barrett's esophagus: updated prospective multivariate analysis. *American Journal of Gastroenterology,* 99**,** 1657-66.

WINTERS, C., SPURLING, T., CHOBANIAN, S., CURTIS, D., ESPOSITO, R., HACKER, J., JOHNSON, D., CRUESS, D., COTELINGAM, J., GURNEY, M. & CATTAU, E. (1987) Barrett's Esophagus. A Prevalent, Occult Complication of Gastroesophageal Reflux Disease. *Gastroenterology,* 92**,** 118-124.

WOLFSEN, H., HEMMINGER, L. & DEVAULT, K. (2004) Recurrent Barrett's esophagus and adenocarcinoma after esophagectomy. *BMC Gastroenterology,* 4.

WONG, D., PAULSON, T., PREVO, L., GALIPEAU, P., LONGTON, G., BLOUNT, P. & REID, B. (2001) p16INK4a Lesions Are Common, Early Abnormalities that Undergo Clonal Expansion in Barrett's Metaplastic Epithelium. *Cancer Research,* 61**,** 8284-8289.

WONG, W., POULSOM, R. & WRIGHT, N. (1999) Trefoil peptides. *Gut,* 44**,** 890-895.

WRIGHT, N., PIKE, C. & ELIA, G. (1990) Induction of a novel epidermal growth factor secreting lineage by mucosal ulceration in human gastrointestinal stem cells. *Nature,* 343**,** 82-85.

ZEKI, S., MCDONALD, S. & GRAHAM, T. (2011) Field cancerization in Barrett's esophagus. *Discovery Medicine,* 12**,** 1371-9.

Electronic Articles

British Society of Gastroenterology (2005) Guidelines for the diagnosis and management of Barrett's columnar-lined oesophagus. Available at: http://www.bsg.org.uk/images/stories/docs/clinical/guidelines/oesophageal/Barretts_Oes.pdf

Cancer Research UK (2012) CancerStats Key Facts. Available at: http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/

National Institute for Health and Clinical Excellence (2004) Management of dyspepsia in adults in primary care. Available at: http://www.nice.org.uk/nicemedia/live/10950/29460/29460.pdf

NHS The information centre for health and social care (2010) National Oesophago-gastric Cancer Audit. Available at: https://catalogue.ic.nhs.uk/publications/clinical/oesophago-gastric/nati-clin-audi-supp-prog-oeso-gast-canc-2010/clin-audi-supp-prog-oeso-gast-2010-rep1.pdf

Office for National Statistics (2012) Summary: Cancer Survival in England: Patients Diagnosed, 2006–2010 and Followed up to 2011. Available at: http://www.ons.gov.uk/ons/dcp171780_283943.pdf