University of Southampton
School of Medicine

Barrett's dysplasia – adenocarcinoma: role of the leukotriene pathway and treatment with radiofrequency ablation

By
Dr Christopher Philip Coryndon Boger
BSc MBChB MRCP

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Abstract

Background

Reflux of acid and bile into the distal oesophagus induces inflammation, which in certain individuals leads to metaplastic cellular changes in the normal squamous cell lining, termed Barrett's oesophagus. Further cellular changes can occur resulting in formation of low grade then high grade dysplastic cells, increasing an individual's risk of progressing to oesophageal adenocarcinoma. The prognosis, once cancer develops, is poor and thus understanding the cellular mechanisms contributing to the progression from squamous through Barrett's metaplasia to cancer, and treatment options for dysplasia to prevent cancer, are important. This thesis explores these two areas, specifically looking at the role of the 5-lipoxygenase (5-LOX) pathway in the progression to oesophageal adenocarcinoma, and the cost-effectiveness of radiofrequency ablation as a new treatment for high grade dysplasia.

The 5-LOX pathway catalyses the biosynthesis of leukotrienes, potent inflammatory mediators. Upregulation of this pathway is found in numerous tumour types and linked to promotion of cancer cell growth, but the role of leukotrienes and their cellular sources in Barrett's metaplasia and oesophageal adenocarcinoma are not fully understood.

If high grade dysplasia is detected at endoscopy then national guidance in the UK recommends oesophagectomy as definitive treatment. Radiofrequency ablation is an alternative new technology with promising early trial results, but whether it represents a cost-effective alternative to oesophagectomy is unknown.

Hypothesis

(1) 5-LOX pathway activity is up-regulated progressively through metaplasia to adenocarcinoma, by induction of enzyme expression and/or infiltration by inflammatory cell-types.
(2) Radiofrequency ablation is a cost-effective option for treatment of high-grade dysplasia in Barrett's in a UK setting.

Methods

Tissue samples were collected at endoscopy from patients with Barrett's metaplasia and oesophageal adenocarcinoma, with proximal squamous oesophagus samples used as controls. The diagnosis was histologically confirmed from adjacent sections by a gastrointestinal pathologist. Immunohistochemical analyses were performed on stromal and epithelial areas (5 squamous, 16 Barrett's, 7 oesophageal adenocarcinoma) with optimized concentrations of primary antibodies for 5-LOX, 5-LOX activating protein (FLAP), and the distal enzymes leukotriene A_4 hydrolase (LTA_4H)

and LTC_4 synthase (LTC_4S). Epithelial and stromal areas of squamous (n=7), Barrett's metaplasia (n=12), and oesophageal adenocarcinoma (n=9) were isolated using laser capture microdissection. mRNA was then extracted, reverse transcribed and real time quantitative polymerase chain reaction (RT-qPCR) assays were performed for the 5-LOX pathway genes *ALOX5, ALOX5AP, LTA4H, LTC4S, LTB4R, CYSLTR1.*

A cost-utility analysis was undertaken using a Markov model to simulate the natural history of a cohort of patients with high-grade dysplasia in Barrett's oesophagus undergoing one of two treatment options: (i) oesophagectomy or (ii) radiofrequency ablation followed by endoscopic surveillance with oesophagectomy for high-grade dysplasia recurrence or persistence.

Results

Significant increases in stromal immunoexpression of 5-LOX pathway enzymes were seen in adenocarcinoma tissue, with correlation of FLAP and LTA_4H immunostaining with elevated neutrophil counts ($p<0.001$). LTC_4S immunostaining was also notably overexpressed within epithelial cells in both Barrett's ($p<0.001$) and adenocarcinoma ($p<0.01$) tissue. Transcript levels for all 5-LOX pathway enzymes were higher in stromal area compared with epithelial areas of all tissue types, but in particular in oesophageal adenocarcinoma tissue. mRNA transcripts of the cysteinyl leukotriene receptor 1 (*CYSLT1R)* and leukotriene B_4 receptor 1 (*LTB4R)* genes were expressed most prominently in Barrett's stromal and oesophageal adenocarcinoma epithelial areas respectively.

In the health economic analysis, radiofrequency ablation dominated as it generated 0.4 extra quality of life years at a cost saving of £1902. For oesophagectomy to be the most cost-effective option required a radiofrequency ablation treatment failure rate (high-grade dysplasia persistence or progression to cancer) of >44%, or an annual risk of high-grade dysplasia recurrence or progression to cancer in the ablated oesophagus of >15% per annum. There was an 85% probability that radiofrequency ablation remained cost-effective at the NICE willingness to pay threshold range of £20,000-30,000.

Conclusions

Key biosynthetic enzymes of the LTB_4 and LTC_4 biosynthetic pathways are incrementally expressed across the spectrum of squamous, Barrett's metaplasia and oesophageal adenocarcinoma tissues, suggesting for the first time a role of both leukotriene sub-families in disease progression. Transcriptional upregulation of mRNA was highest in the stromal area of oesophageal adenocarcinoma tissue, although marked sample to sample variation was observed, suggesting an important role for post-transcriptional factors of 5-LOX pathway protein translation. Expression of *LTB4R* by epithelial cells of

oesophageal adenocarcinoma tissue infers a functional role for LTB_4 acting on BLT1 receptors in carcinogenesis.

In the prevention of oesophageal adenocarcinoma, radiofrequency ablation is likely to be a cost-effective option for treating high-grade dysplasia in Barrett's oesophagus in a UK setting. Long term outcome data are required to achieve certainty as to the optimal management strategy. However, if initial eradication of high-grade dysplasia is achievable in over 60% of patients, then radiofrequency ablation with oesophagectomy for high-grade dysplasia persistence or recurrence will be the preferred strategy.

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Declaration of Authorship

I, Dr Christopher Philip Coryndon Boger

declare that the thesis entitled

Barrett's dysplasia – adenocarcinoma: role of the leukotriene pathway and treatment with radiofrequency ablation

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

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:

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Signed: ……

Date:……

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Commonly used Abbreviations

OA – oesophageal adenocarcinoma
BM – Barrett's metaplasia
GORD – gastro-oesophageal reflux disease
LGD – low grade dysplasia
HGD – high grade dysplasia
NHS – National Health Service
RFA – radiofrequency ablation
UK – United Kingdom
IL - interleukin
DNA – deoxyribonucleic acid
RNA – ribonucleic acid
COX – cyclo-oxygenase
NF-κβ - nuclear factor kappa β
TGFβ – transforming growth factor β
AA – arachidonic acid
LT - leukotriene
5-LOX – 5-lipoxygenase
FLAP – 5-lipoxygenase activating protein
LTA_4H – leukotriene A_4 hydrolase
LTC_4S – leukotriene C_4 synthase
PG – Prostaglandin
cysLT – cysteinyl leukotriene
NSAID – non-steroidal anti-inflammatory drug
RCT – randomised controlled trial
RT-qPCR – real time quantitative polymerase chain reaction
EMR – endoscopic mucosal resection
PDT – photodynamic therapy
VAS – visual analogue score
NICE – national institute for Health and Clinical Excellence
PPI – proton pump inhibitor
LCM – laser capture microdissection
ICER – incremental cost-effectiveness ratio
QALY – quality of life
IHC – immunohistochemistry
AEC - 3-amino-9-ethylcarbazole
DAB - 3, 3'-diaminobenzidinetrahydrochloride
GM – geometric mean

CHAPTER 1: Introduction

This thesis is submitted in consideration of a Doctorate of Medicine award. It outlines the findings of several studies on aspects of Barrett's oesophagus and oesophageal adenocarcinoma. This introductory chapter describes the development and context of the research. Chapter two concerns the methodology of the laboratory studies and protocol. Chapter three outlines the methodology of the health economic analysis, including literature review, model design, and formulation of data parameters and costs. Results and discussion of the studies are presented in chapters four through six, with final conclusions and scope for future study presented in chapter seven.

1.1 Overview

The incidence of oesophageal adenocarcinoma (OA) is highest in the United Kingdom (UK) and rising faster than any other solid tumour in western countries (Blot et al. 1991; Jankowski et al. 2002). It is associated with a poor prognosis, with a 5-year survival of 10-13% (Rachet et al. 2009; Sant et al. 2009). OA arises in metaplastic columnar epithelium in the distal oesophagus, named Barrett's metaplasia (BM). BM confers a 1 in 20 life time risk of developing OA, which equates to an increase of between 30 to 125 times greater than that of the general population (Provenzale et al. 1999; Wild and Hardie 2003). BM is estimated to be prevalent in 0.5-2% of adults in the western world, rising to up to 15% in those with chronic gastro-oesophageal reflux disease (GORD), suggesting that GORD is an important risk factor (Corder et al. 1996; Csendes et al. 2000; Ronkainen et al. 2005). Reflux of acid and bile results in an inflammatory injury to the lower oesophagus, which leads to the development of BM, and subsequent OA in a proportion of patients (Jankowski and Anderson 2004).

Chronic inflammation is a well-established risk factor for cancer, providing a micro-environment rich in molecules which enhance cell growth and survival (Coussens and Werb 2002; Aggarwal et al. 2006; Lu et al. 2006). 5-lipoxygenase (5-LOX) and its constituent pathway proteins metabolise arachidonic acid to leukotrienes, potent pro-inflammatory mediators (Funk 2001). Up-regulation of this pathway is evident in a variety of tumour types and has been shown to play a role in promotion of cancer cell growth (Romano and Claria 2003). The 5-LOX pathway is increased in oesophagitis, BM and OA, but their role has yet to be fully elucidated (Triadafilopoulos et al. 1996, Chen et al. 2002).

Due to the dismal prognosis associated with OA, efforts have focused on detection of BM in high risk groups and subsequent 2-3 yearly surveillance (Watson et al. 2005). At this juncture, endoscopy is performed and multiple biopsies are taken with the aim that early precancerous changes (dysplasia) are detected, signalling the need for more intensive surveillance or intervention. The development of high-grade dysplasia (HGD) within a Barrett's segment is associated with progression to invasive cancer in 30-35% within 5-years (Rastogi et al. 2008). The recommended management of patients in the UK with persistent HGD, despite intensive acid suppression, is oesophagectomy (Watson et al. 2005). However, surgical oesophagectomy is a high-risk procedure, even in tertiary centres, with a 30-day mortality rate of 3.2%, significant post-operative complications in up to 40%, and long-term morbidity (Palser et al. 2009). Oesophagectomy has an approximate cost to the National Health Service (NHS) of £7500/person.

A new, alternative management strategy for patients with HGD in BM is radiofrequency ablation (RFA) using the HALO device. Results from a randomised sham-controlled study have shown that it is effective at eliminating HGD in BM in up to 90% of patients at 12 months follow up, with minimum treatment-related side effects or reduction in quality of life (Shaheen et al. 2008a; Shaheen et al. 2009). Furthermore there is complete reversion to squamous oesophageal lining in 80% of patients. The cost per patient to the NHS is currently unknown and to date there has been no randomised controlled trial comparing RFA and oesophagectomy outcomes.

The aim of this thesis was thus two-fold; firstly to explore the expression of the 5-LOX pathway proteins in BM and OA, and secondly to evaluate the health economic impact on the introduction of RFA for the treatment of HGD in the UK.

1.2 Definition of Barrett's metaplasia

BM is visualised and suggested by findings at endoscopy, which reveal that the normal paler lining of the oesophagus, has been replaced by salmon pink mucosa, extending proximally from the gastro-oesophageal junction (Figure 1-A). Confirmation is by histological evaluation of biopsy samples from the affected area of the oesophagus, which reveal characteristic columnar epithelium with a brush border and goblet cells, termed specialised intestinal metaplasia. Although the definition of Barrett's as above is now accepted, there has been great controversy and debate to reach this clarity.

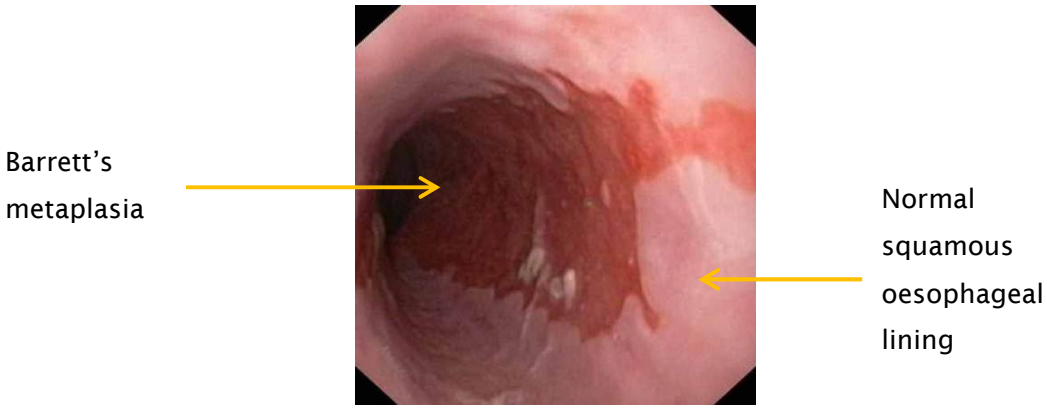

Figure 1-A: Appearance of Barrett's oesophagus at white light upper gastrointestinal endoscopy.

1.3 Historical perspective

In 1906 an American pathologist named Tileston described several patients with peptic ulcer of the oesophagus, commenting on "the close resemblance of the mucous membrane about the ulcer to that found in the stomach" (Tileston 1906). Further similar observations were made and then in 1950, Norman Barrett, a surgeon working at St. Thomas's Hospital, London, wrote a paper in the British Journal of Surgery describing a 'columnar lined intra-thoracic structure' (Barrett 1950). At this point Barrett, along with a number of others, argued that this was likely to represent a tubular segment of stomach, pulled into the thorax by a congenitally short squamous oesophagus. In 1953, Allison and Johnstone correctly interpreted 'Barrett's oesophagus' as being due to metaplasia of the normally squamous oesophageal mucosa (Allison and Johnstone 1953). However, it was not until 1957 that Barrett revised his opinion and conceded this point, although at that stage he felt the most likely explanation was that of failure of the embryonic lining of the gullet to reach normal maturity (Barrett 1957). This explanation was widely accepted until Hayward published his own opinions. He summarised by stating that the changes in the lower oesophagus were ".....probably neither ectopic, nor congenital, nor permanent, nor in need of resection but metaplastic and reversible" (Hayward 1961).

A detailed study of manometric guided oesophageal biopsies published in 1976 described the histological features of the Barrett's segment (Paull et al. 1976). They described the more proximal segment of the columnar lined epithelium as displaying a "distinctive specialised columnar epithelium with a villiform surface, mucous glands and intestinal-type goblet cells". This is now accepted this as the histological diagnostic feature of Barrett's oesophagus (Fléjou 2007).

The finding of OA in association with intestinal-type mucosa containing goblet cells was described in a case report by Morson and Belcher (1952), although at that point they still felt they were dealing with a congenitally short oesophagus. Further observations in oesophagectomy specimens in the 1960's and 1970's cemented the association between Barrett's oesophagus and OA (Adler 1963; Hawe et al. 1973; Haggitt et al. 1978). By the late 1980's it was appreciated that specialised intestinal metaplasia in particular predisposed to OA (Reid and Weinstein 1987).

1.4 Epidemiology of Barrett's oesophagus and oesophageal adenocarcinoma

The incidence of OA has risen inexorably over the last two decades, so that now it accounts for over 50% of histological subtypes (Lagergren 2005). In the 1970s, adenocarcinoma of the oesophagus accounted for less than 5% of oesophageal malignancies, with squamous cell carcinoma of the oesophagus accounting for the majority. The precise reasons for the rapid rise are unknown, but of importance is the paralleled increase in BM, now acknowledged to be the precursory lesion for OA.

1.4.1 Prevalence of Barrett's oesophagus and epidemiological evidence linking reflux with Barrett's

BM is thought to be resultant from chronic GORD which is present in up to 18% of the UK population (Lagergren et al. 1999; Dent et al. 2005). Longer duration and increased frequency of symptoms of reflux are important risk factors for development of BM (Eisen et al. 1997; Conio et al. 2002). BM *per se* is asymptomatic and thus exact true prevalence is unknown. Estimates range widely depending on the study population. For example, a study in Sweden undertook gastroscopy on a representative random subsample of 1000 of the adult population and found BM present in 1.6% overall, with a higher prevalence of 2.3% in those with reflux symptoms (Ronkainen et al. 2005). Other studies have found higher prevalence figures, but the populations have been different. For example, a prevalence of 25% was found in 108 mainly male patients who underwent a gastroscopy, whilst attending for flexible sigmoidoscopy for bowel cancer screening purposes (Gerson et al. 2002). Another study undertook a gastroscopy in 931 patients attending for colonoscopy for all indications (Rex et al. 2003). In this group the prevalence was 6.8% overall, again with a higher prevalence of 8.3% in those with reflux symptoms. Although differing prevalence figures may be accounted for by different population demographics, a finding mirrored in all these studies is the higher prevalence rate of BM amongst those with symptomatic reflux.

Factors that increase an individual's chances of GORD may also be important. These include increasing body mass index, and eradication of Helicobacter pylori (El-Serag et al. 2007; Corley et al. 2008). Body mass index is additionally an independent risk factor for the development of OA, although the mechanisms are not clear (El-Serag 2008). Medications that relax the lower oesophageal sphincter, such as aminophylline and calcium channel blockers could theoretically increase an individual's risk for GORD and thus BM and OA, but the literature is conflicting (Lagergren 2006, Vaughan 1998).

1.5 The role of acid reflux and inflammation in the pathogenesis of Barrett's Metaplasia and Oesophageal Adenocarcinoma

The understanding of the malignant degeneration to OA has advanced and the consensus is that it is a multistep process, in which BM progresses through worsening stages of dysplasia to early carcinoma, and then eventually invasive cancer. This is termed the metaplasia-dysplasia-adenocarcinoma sequence (Jankowski et al. 1999).

1.5.1 Reflux and inflammation

Chronic acid and bile reflux into the lower oesophagus with resulting inflammation appear to be important initiating events in BM and the progression to OA. Along with the epidemiological evidence described earlier, studies have demonstrated that patients with both BM and early OA have more frequent and longer periods of both oesophageal acid and bile salt exposure when compared with healthy controls or those with uncomplicated GORD (Iascone et al. 1983; Vaezi and Richter 1996; Niemantsverdriet et al. 1997; Stein et al. 1998). This exposure of the lower oesophagus to the gastro-duodenal refluxate induces injury to the oesophageal mucosa, and a subsequent inflammatory response. The mechanism by which this injury triggers metaplasia, and why this occurs in some but not all individuals, is unknown.

1.5.2 Linking inflammation and cancer

Inflammation and cancer have a well-recognised association, first described by Virchow in 1863, and now demonstrated in a variety of tissue types (Macarthur et al. 2004). In the gastrointestinal tract there is an association between chronic inflammation in the colon seen with inflammatory bowel disease and increased risk of colorectal dysplasia and OA (Vagefi and Longo 2005). Chronic hepatitis C virus infection and resultant hepatic inflammation predisposes to hepatocellular carcinoma (Di Bisceglie 1997). Metaplasia occurs in the context of chronic inflammation in other tissue sites, carrying an increased risk of cancer (Slack 2000). For example, gastric Helicobacter pylori

infection may result in chronic inflammation and subsequent intestinal metaplasia and cancer (Akiyama and Uemura 2009). In these examples it is the potent mix of inflammatory mediators, such as cytokines, chemokines, lipids, reactive oxygen species (ROS), and metalloproteinases, that are thought to be important in the tumorigenic process, through promotion of growth, suppression of apoptosis, angiogenesis, invasion and metastasis (Coussens and Werb 2002).

1.5.3 Inflammation leads to deoxyribonucleic acid damage

Inflammation in GORD induces oxidative stress, and increased levels of ROS are found in BM, with an associated decrease in antioxidant defences (Olyaee et al. 1995). Exposure of BM and OA cell lines to components of the refluxate further increases formation of ROS. This in turn leads to subsequent deoxyribonucleic acid (DNA) damage through induction of double stranded DNA breaks, which promotes genomic instability and may lead to carcinogenesis (Wiseman and Halliwell 1996; Clemons et al. 2007). It is the progressive accumulation of genetic and epigenetic aberrations that is believed to lead to one or more clones with malignant potential. Many genetic abnormalities have been reported in patients with BM and OA, such as gene mutations, gene deletions, loss of heterozygosity (LOH), aberrant methylation and aberrant gene expression (McManus et al. 2004).

Aneuploidy (abnormal number of chromosomes) reflects widespread DNA changes due to genomic instability (Morales et al. 2002). It is found in over 90% of HGD and OA, and may predict progression to cancer in those with BM (Reid et al. 1992). Loss of p53 function is a common genetic abnormality seen in the metaplasia-dysplasia-adenocarcinoma sequence. The p53 gene is a cell-cycle control gene that prevents cells with genome damage from entering DNA synthesis during which the breaks could be replicated, cause chromosome damage, and lead to progressive genetic instability and cancer. Mutation causing loss of one allele of the p53 gene occurs in up to 60% with BM, and up to 66% with HGD (Altorki et al. 1997). Loss of further genetic information as a result of deletion of a portion of the chromosome or other events is termed LOH, and results in gene inactivation. In a study by Reid et al., 77% of those with BM who progressed to cancer had LOH at baseline endoscopy, suggesting a possible role for it as a biomarker (Reid et al. 2001).

Abnormalities in the expression of cyclins are found through the metaplasia-dysplasia-adenocarcinoma sequence. Cyclins are promoters of cell-cycle progression through their ability to phosphorylate retinoblastoma protein after complexing with cyclin-dependent kinases (CDK) (Weinberg 1995). For example, expression of cyclins D1 and E increases with worsening degrees of dysplasia. In addition, LOH and promoter

hypermethylation of the CDK inhibitor, p16, occurs in up to 80% of patients with BM, and p16 hypermethylation correlates with the degree of dysplasia in intestinal metaplasia (Eads et al. 2000; Kawakami et al. 2000).

1.5.4 Mediators of Inflammation in Barrett's adenocarcinoma

In other premalignant conditions that develop in the setting of chronic inflammation there are associated changes in the inflammatory response, such as down-regulation of cell-mediated immunity (Th1) and the presence of a more humoral (Th2) infiltrate (Coussens and Werb 2002). Similarly, BM is associated with a more pronounced Th2 response compared with reflux oesophagitis. The total numbers of inflammatory cells increase, with the relative proportion of plasma cells increased, whilst the proportion of macrophages and CD8+ cytotoxic T-cells decreases (Moons et al. 2005). These infiltrating inflammatory cells contribute to a highly complex inflammatory microenvironment, rich in biological mediators with pro-tumour effects.

Differences in cytokine profile exist between oesophagitis, BM and OA. In oesophagitis, IL-1β, IL-8 and interferon (IFN) -γ are increased compared to BM, even in the presence of continuing reflux (Fitzgerald et al. 2002b).

An inflammatory gradient exists within BM, with inflammation maximal in areas close to the squamo-columnar junction, characterised by elevation in the pro-inflammatory cytokines IL-1β and IL-8 (Fitzgerald et al. 2002a). More distally, less inflammation is observed, suggesting that factors other than acid must be responsible for driving the inflammatory response, as acid exposure reduces proximally in the oesophagus. The anti-inflammatory cytokine IL-10 is elevated distally. This is particularly interesting as IL-10 can also influence tumour growth by affecting cell proliferation and angiogenesis, through a variety of pathways including up-regulation of signal transducers and activators of transcription (STAT3), and down-regulation of cyclo-oxygenase (COX) -2 and vascular endothelial growth factor (VEGF) (Mocellin et al. 2005). Other cytokines are also implicated. IL-6, a cytokine associated with a Th2 response, is increased in BM compared to normal oesophagus, and has an anti-apoptotic role through STAT3 (Dvorakova et al. 2004). Interestingly, this pathway was induced by short exposure of OA cells to an acid/bile mix at low pH, again providing evidence for the important role of acid/bile reflux (Dvorak et al. 2007).

1.5.5 The role of nuclear factor kappa β

The transcription factor nuclear factor kappa β (NF-κβ) is an important regulator of genes that control cell proliferation and cell survival. It is increased progressively through the sequence of normal oesophagus, oesophagitis, BM, and OA (Lee et al.

2001; Konturek et al. 2004). Activated NF-κβ stimulates IL-1β and IL-8, which in turn feedback to increase NF-κβ. NF-κβ is involved in the regulation of a multitude of mechanisms that contribute to cancer development, such as anti-apoptosis, inflammatory genes, oncogenes and angiogenesis factors (Chen et al. 1999). In particular it can promote the transcription of *COX-2*, *c-myc* and *cyclin D1*. Additionally, NF-κβ can induce caudal type homeobox transcription factors (cdx) 1 and 2, key regulators of intestinal epithelial cell phenotype, and thought to be important in the development of BM (Colleypriest et al. 2009).

The expression of TNF-α, one of the major mediators of inflammation, is regulated by NF-κβ. TNF-α has been linked to all steps of tumorigenesis by mediating proliferation and by inducing invasion and angiogenesis (Aggarwal et al. 2006). Levels of TNF-α increase along the metaplasia-dysplasia-adenocarcinoma sequence, and can induce expression of the transcription factor c-myc via β-catenin signalling in columnar epithelial cell lines (Tselepis et al. 2002). This action was independent of NF-κβ.

1.6 Eicosanoids in cancer

Eicosanoids are lipid mediators involved in modulating the intensity and duration of inflammatory and immune responses. Arachidonic acid (AA) is a polyunsaturated fatty acid (PUFA) obtained through the diet or by conversion from the *n-6* PUFA linoleic acid (the main PUFA in human diets) by chain elongation and then desaturation. It is found esterified within phospholipids such as phosphatidylinositol by the action of acyl transferases on both the plasma membrane and in intracellular membranes. Only low levels are present in the cytosol, but hydrolysis of membrane phospholipids by stimulated lipases produces free AA, which may then serve as a substrate for cytochrome P450, cyclooxygenase (COX) and lipoxygenase (LOX) enzymes (Funk 2001). AA is of particular importance as it is the precursor substrate for eicosanoid synthesis.

COX catalyses the formation of AA to prostaglandin (PG) H_2, the immediate precursor of biologically important lipid mediators, the prostanoids, which include the prostaglandins and thromboxane. The prostanoids have diverse roles coordinating cellular signalling by binding trans-membrane G-protein coupled receptors. These range from the regulation of vascular tone, permeability, and platelet function to the induction of hyperalgesia and fever.

1.6.1 Cyclo-oxygenase

COX has two distinct isoforms. COX-1 is constitutively expressed in most tissues, and plays an important role in platelet aggregation and gastric cytoprotection. Although COX-2 is expressed constitutively in the kidney and brain, in many tissues it is only induced in response to inflammation, wound healing, and neoplasia. This pathway is clinically relevant, as it is the main target for non-steroidal anti-inflammatory drugs (NSAIDs), COX-2 selective inhibitors (coxibs), and aspirin (see figure 1-B). Therapeutic concentrations of NSAIDs and aspirin are not known to influence other pathways of AA metabolism except indirectly by increasing the intracellular concentration of free AA, which potentially causes shunting of arachidonic acid through other metabolic pathways, such as the LOX pathway.

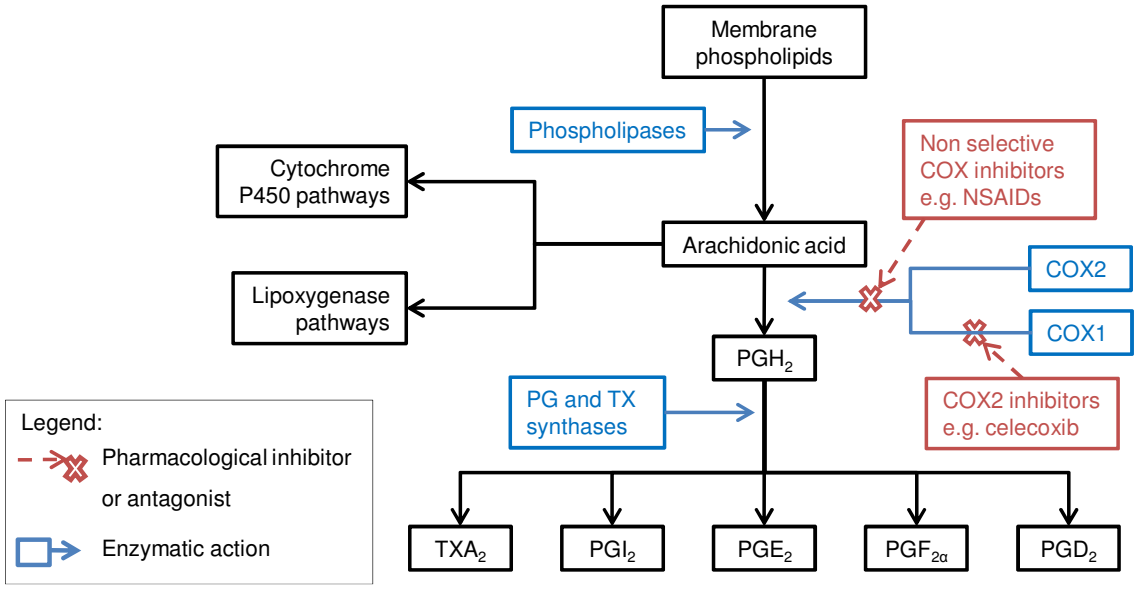

Figure 1-B: Cyclo-oxygenase pathway with examples of available pathway protein inhibitors.
Abbreviations; COX – cyclo-oxygenase, PG – prostaglandin, TX – thromboxane, NSAID – non-steroidal anti-inflammatory drug

1.6.2 Evidence for the role of eicosanoids in cancer

A direct role for eicosanoids in cancer was first suggested by studies in animals showing that dietary fats enhance carcinogenesis. In particular, *n-6* PUFAs are associated with promotion of tumorigenesis, whereas *n-3* PUFAs have anti-tumorigenic effects (Broitman et al. 1977; Singh et al. 1997; Chang et al. 1998). A key epidemiological study by Kune *et al*. then showed that subjects using NSAIDs for various indications had a significantly lower incidence of colon cancer (Kune et al. 1988), a finding since confirmed by numerous epidemiological studies (Thun et al. 2002). NSAID use is also associated with a lower incidence of cancers at other sites

including the oesophagus (Thun et al. 1993). Randomised controlled trials (RCT) in patients with familial adenomatous polyposis have shown that NSAIDs, including the COX-2 specific inhibitor, celecoxib, can decrease the number or cause complete regression of colonic polyps (Nugent et al. 1993; Steinbach et al. 2000). However, there has been conflicting evidence in other clinical trials. A RCT of male physicians investigated the effects of aspirin versus placebo administered every other day for 5 years with a planned 12-year follow up (Steering Committee of the Physicians' Health Study Research Group 1989). The study terminated after 5 years with no evidence of reduction in incidence of colorectal cancer or reduction in colorectal cancer mortality. These studies have also been hampered by treatment-related side effects of gastrointestinal ulceration, and increased risk of acute myocardial infarction in those on COX-2 specific inhibitors, which have raised questions over the tolerability of their long term use. The subsequent finding that the cardiovascular side-effects may be due to inhibition of endothelial cell-derived COX-2 activity and prostacyclin generation highlights the importance of having a full understanding of the cellular mechanisms underlying drug effects before embarking on large, population-based studies.

The role of COX-2 in the formation of OA has been extensively studied. Acid and bile can up-regulate COX-2 expression and lead to an increase in prostaglandin (PG) E_2 (Shirvani et al. 2000; Kaur and Triadafilopoulos 2002). Selective inhibition of COX-2 leads to a reduction in the proliferation of Barrett's epithelial and OA cells (Souza et al. 2000; Buttar et al. 2002). Whether COX-2 is constitutively expressed in BM and OA is open to debate. A systematic review incorporated 27 studies that assessed COX-2 protein or gene expression in BM, dysplastic or adenocarcinoma tissue (Mehta et al. 2006). There was considerable variability between studies with some studies showing no or only very low levels of expression in BM and dysplasia. COX-2 was generally found to be expressed in OA, although there was considerable variation in the levels of its expression.

A meta-analysis by Corley et al. summarised the observational studies evaluating the association between aspirin, NSAIDs, and oesophageal cancer (Corley et al. 2003). Nine studies were included containing a total of 1813 cancer cases. The findings of this study were that any use of aspirin or NSAID was associated with a 33% reduction in the odds of developing OA. There appeared to be a greater effect with aspirin (50% reduction) compared with NSAIDs (25% reduction), and also a dose-dependent effect, with frequent use associated with a 46% reduction of developing either squamous cell or adenocarcinoma of the oesophagus. The findings of this meta-analysis imply a protective effect of regular aspirin or NSAID use in oesophageal cancer. However, there are a number of limitations of this study which include the use of observational data,

lack of clarity of dosing, and the lack of differentiation between squamous cell and adenocarcinoma of the oesophagus. Thus definite conclusions cannot be reached. The on-going Aspirin Esomeprazole Chemoprevention (AspECT) study hopes to clarify this. In this RCT, patients have been randomised to receive acid suppression using the proton pump inhibitor (PPI) esomeprazole with or without aspirin. The final analysis of this multicentre study is expected in 2016.

Initially it was felt that COX-2 was the major target of NSAID effects on colorectal and other cancers, but the discrepancy in the published studies point towards the involvement of other molecular targets. Numerous COX-independent targets have been demonstrated including inhibition of NF-κβ, extracellular-signal-regulated kinases 1/2 (ERK1/2), Wnt signalling including c-myc, cyclin D1 and peroxisome proliferator-activated receptor-γ (PPAR-γ) and PPAR-δ, and proteins of the lipoxygenase family (Kashfi and Rigas 2005).

1.7 The 5-lipoxygenase pathway

1.7.1 Source, cellular localisation, and biosynthesis of leukotrienes

5-LOX is a non-haem dioxygenase which catalyses the biosynthesis of leukotrienes (LTs). It is a 674 amino acid, 78 kilodalton (kDa) protein, consisting of an amino acid terminal, a β barrel domain capable of binding two calcium ions and protein kinase C, and a carboxy (catalytic) terminal domain (Funk 2001). Additional alpha helices hold iron through histidine and isoleucine residues. The human 5-LOX gene (*ALOX5*) is located to chromosome 10q11.2 and is over 82 kilo-base pairs (kbp), containing 14 exons (Funk et al. 1989). Optimal activity of 5-LOX requires cofactors calcium, adenosine-5'-triphosphate, 5-LOX activating protein (FLAP), and additional oxidation of the ferrous iron of the resting form to the ferric form by lipid hydroperoxide.

FLAP, an 18kDa protein containing 161 amino acids, is integral to the nuclear envelope and facilitates the docking of AA to 5-LOX (Funk 2001). The human FLAP gene (*ALOX5AP)* is located on chromosome 13q12 and is over 31 kbp, containing 5 exons (Kennedy et al. 1991). Significant expression of 5-LOX and FLAP is confined to bone marrow derived cells, including neutrophils, eosinophils, monocytes/macrophages, mast cells, certain lymphocyte populations, and dendritic cells. Additionally, lower level expression has been documented in other cells and tissues, such as epithelial cells, fibroblasts, and in various parts of the brain (Peters-Golden and Brock 2003).

In metabolically resting cells, 5-LOX is distributed throughout the cytoplasm and nucleus. On cellular activation 5-LOX translocates to, and/or across the nuclear

envelope. On either side of the nuclear envelope 5-LOX encounters arachidonate-specific phospholipase A_2 (PLA_2) and FLAP. PLA_2 hydrolyses membrane phospholipids liberating AA and donating it to FLAP. The crystal structure of FLAP reveals a homotrimer, with the transmembrane region of each monomer providing a lipophilic pocket for the binding of AA (or of FLAP inhibitors) (Ferguson et al. 2007; Evans et al. 2008). FLAP donates the AA to 5-LOX, which catalyses its oxygenation to 5-S-hydroperoxyeicosatetraenoic acid (5-S-HPETE). This can be reduced non-enzymatically to 5-hydroxyeicosatetraenoic acid (5-S-HETE) or transformed by 5-LOX to a highly unstable allylic epoxide, leukotriene A_4 (LTA_4). LTA_4 has three possible fates depending on the cellular context.

Firstly, as occurs preferentially in neutrophils and monocytes, hydrolytic attack by LTA_4 hydrolase (LTA_4H) in the cytoplasm and possibly the nucleus adds an hydroxyl group at the 12 position to generate a dihydroxylated product, leukotriene B_4 (LTB_4; 5,12-dihydroxy-eicosatetraenoic acid). LTB_4 may interact with intracellular PPAR receptors, or be exported from the cell by an as yet unknown transporter to act at extracellular receptors. Secondly, as occurs preferentially in mast cells, eosinophils, and to a lesser extent in monocytes and macrophages, conjugation with glutathione catalysed by leukotriene C_4 synthase (LTC_4S) produces leukotriene C_4 (LTC_4). LTC_4 is transported out of the cell by transporters such as the multi-drug resistance protein (MRP)-1. LTC_4 may be converted extra-cellularly to leukotriene D_4 (LTD_4) by gamma-glutamyl transpeptidase, and then to leukotriene E_4 (LTE_4) by a dipeptidase. LTE_4 is excreted in the urine. Collectively LTC_4, D_4, and E_4 are termed the cysteinyl leukotrienes (cysLTs). Lastly, transcellular metabolism may occur to generate bioactive eicosanoids. For example, LTA_4 generated by leukocytes may be exported into endothelial cells expressing LTA_4 or LTC_4S, and thus be converted to LTB_4 or LTC_4 (Fabre et al. 2002).

LTA_4H is a 69 kDa soluble monomeric protein containing 610 amino acids, with bi-functional enzymatic activity; epoxide hydrolase (LTA_4 to LTB_4) and peptide-cleaving activity (Haeggstrom 2000). The human LTA_4H gene (*LTA4H*) is located on chromosome 12q22 and is over 35 kilo-base pairs (kbp), containing 19 exons (Mancini and Evans 1995). It resides in the cytosol of a myriad of organs, tissues and individual cell types. In the blood, neutrophils, monocytes, lymphocytes, and erythrocytes are rich sources. In contrast, eosinophils have low levels and basophils and platelets seem to be virtually devoid of the enzyme.

LTC_4S is an 18 kDa protein, containing 150 amino acids, with synthase enzymatic activity (Lam and Austen 2002). It is found in cells exclusively of haematopoietic origin, specifically eosinophils, basophils, mast cells, macrophages/monocytes, and platelets,

where it is localised to the nuclear envelope and adjacent endoplasmic reticulum. The human LTC_4S gene (*LTC4S*) is located to chromosome 5q35 and spans 2.5 kbp, containing 5 small exons (Penrose et al. 1996).

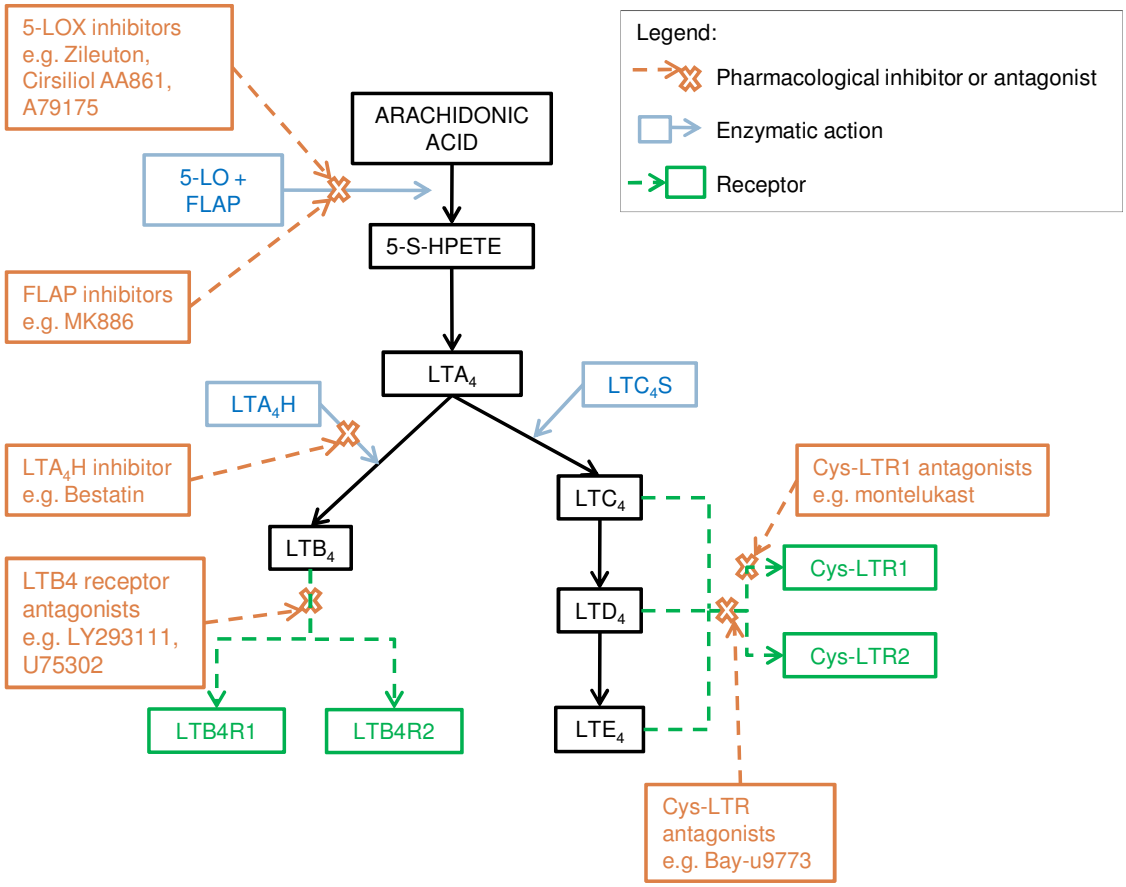

Figure 1-C: 5-LOX pathway with examples of available pathway protein inhibitors or receptor antagonists

1.7.2 Mechanisms of action

LTs exert their actions through one or more of at least four distinct hepto-helical receptors located on the outer membrane of structural and inflammatory cells, the leukotriene B receptors 1 and 2 (BLT1 and BLT2) and the cysteinyl- leukotriene receptors 1 and 2 (cysLT1R or cysLT2R). These G-protein coupled receptors initiate calcium-dependent or –independent activation of downstream kinase cascades and thus alter cellular activity.

The BLT1 and BLT2 receptors are products of the *LTB4R1* and *LTB4R2* genes located on chromosome 14q11.2-q12. The *LTB4R2* locus is approximately 3kb 5' of the *LTB4R1* gene (Nilsson et al. 2000), such that the third exon of *LTB4R2* overlaps with the promoter region and first exon of *LTB4R1*. The BLT1 receptor is expressed mainly on

leucocytes, in particular neutrophils, macrophages, and eosinophils, although a murine T cell lymphoma model suggested low levels of BLT1 may be expressed by T-cells (Huang et al. 1998). In contrast, BLT2 expression is present in most human tissues, with expression highest in spleen, liver, ovary and peripheral blood leucocytes. LTB_4 through the BLT1 receptor plays a key role in leukocyte recruitment to sites of inflammation, both by directing cellular migration and by facilitation of endothelial cell binding, a prerequisite of leukocyte migration into tissues (Tager and Luster 2003). In addition LTB_4 may prolong neutrophil survival by preventing apoptosis (Hebert et al. 1996). LTB_4 has lower affinity for BLT2 receptors than for BLT1, but is shown to mediate similar, calcium-dependent signalling and cellular responses. A clearer role for this low-affinity receptor has yet to be defined, although unlike BLT1, which is highly-selective for LTB_4, the BLT2 receptor also binds some other lipoxygenase products including 12-HETE and 15-HETE (Yokomizo et al. 2001). A summary of the roles of LTB_4 is shown in figure 1-D.

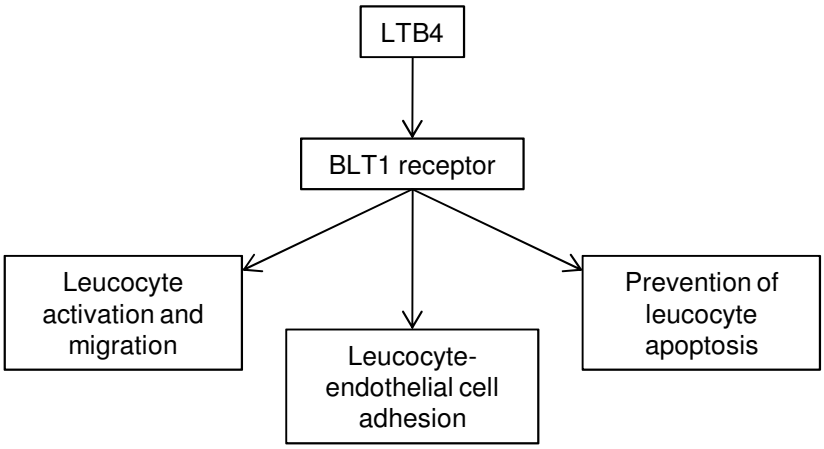

Figure 1-D: Actions of LTB_4

The cys-LTs are thought to act in humans mainly through the cysLT1 and cysLT2 receptors, which are analogous to the LTC_4 receptor and the LTD_4 receptor identified in rodents, respectively. A human LTC_4/LTD_4 receptor, known as GPR17, has also been identified in human brain (Ciana et al. 2006). An LTE_4-selective receptor ($CysTL_E$) has also been described in rodents (Maekawa et al. 2008). In humans, LTE_4 has some specific effects not shared with the other cysLTs, such as eosinophilotaxis, and LTE_4 is now recognised as a potent ligand of the $P2Y_{12}$ purinoceptor, the target of clopidogrel and related anti-platelet drugs (Paruchari et al.2009). There is increasing recognition of the family relationships between cysLT receptors and purinoceptors and other examples of cross-affinity for cys-LT and purine ligands may emerge.

The gene encoding the cysLT1 receptor (*CYSLTR1)* is located to chromosome Xq13.2-q21.1 and consists of at least 3 exons (Lynch et al. 1999). The gene encoding the cysLT2 receptor (*CYSLTR2)* is located to chromosome 13q14.12-q21.1 and consists of a single exon (Heise et al. 2000). CysLT1R is found mainly on airway smooth muscle cells and vascular endothelial cells, but additionally may be found on peripheral leukocytes (mast cells, eosinophils, neutrophils, monocytes and macrophages). CysLT2R is expressed in smooth muscle cells, peripheral blood leukocytes (monocytes and eosinophils), lymph nodes, spleen, heart, and the central nervous system.

Cys-LTs were initially discovered to be potent bronchoconstrictors ('slow-reacting substance of anaphylaxis') and play a role in asthma exacerbations (Holgate et al. 2003). Subsequently a wide variety of roles have been demonstrated. In the inflammatory response, they act through cysLT1R to mediate activation of leukocytes and chemotaxis (Kanaoka and Boyce 2004). A variety of cytokines may play a role in alteration of cysLT receptor expression. For example, IL-4 and IL-13 can up-regulate cysLT1R in human peripheral monocytes (Thivierge et al. 2001). There is also evidence that cys-LTs may play an autocrine or paracrine role in the production of cytokines, for example stimulating IL-4 primed mast cells to produce cytokines such as IL-5 and TNF-α (Mellor et al. 2002). In animal models of inflammation additional roles have been demonstrated including induction of airway remodelling (smooth muscle hyperplasia and subepithelial hyperplasia) and increases in microvascular permeability resultant in protein leakage. A summary of the role of the cys-LTs is illustrated in Figure 1-E.

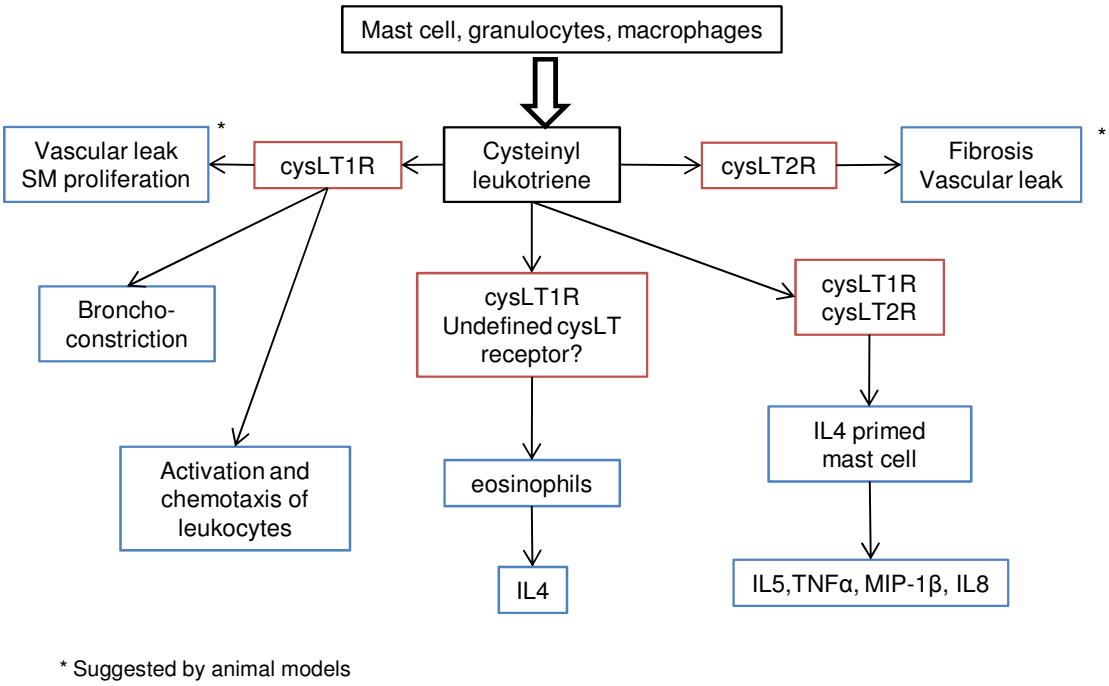

Figure 1-E: Summary of known actions of the cysteinyl leukotrienes mediated through cysLT1 and 2 receptors

1.7.3 The 5-Lipoxygenase pathway in disease

In addition to their role in orchestrating facets of inflammation, products of the 5-LOX pathway are implicated in a number of disease processes. Perhaps the most documented is the role of cys-LTs in asthma exacerbations, inducing potent bronchoconstriction when administered *in vivo*. Increased cys-LTs are found in broncho-alveolar lavage fluid and urine in subjects experiencing acute asthma exacerbations and also after specific allergen challenge of atopic subjects. Clinical efficacy in the treatment of both intrinsic and extrinsic asthmas has also been demonstrated with leukotriene synthesis inhibitors (LTSI), such as zileuton which inhibits 5-LOX, and with antagonists of cysLT1R, such as montelukast (Kanaoka and Boyce 2004). Evidence from a mouse model of asthma demonstrated that CysLT1R antagonists can inhibit the airway remodelling processes (Holgate et al. 2003).

A role for the 5-LOX pathway in coronary artery disease has also been demonstrated. Studies have shown the presence of 5-LOX, FLAP, and LTA_4H in human atherosclerotic vessels (Spanbroek et al. 2003; Qiu et al. 2006), and variations in both 5-LOX and FLAP genotypes can confer increased risk of atherosclerosis and myocardial infarction (Dwyer et al. 2004; Helgadottir et al. 2004). A RCT of a FLAP inhibitor in patients with

known specific at-risk variants of the 5-LOX pathway showed a significant dose-dependent suppression of biomarkers associated with an increased risk of myocardial events (Hakonarson et al. 2005).

1.7.4 The role of 5-lipoxygenase pathway in Cancer

A role for the 5-LOX pathway in cancer was first suggested by Tsukada et al. who demonstrated that specific 5-LOX inhibitors showed potent antiproliferative effects on human leukaemic cell lines (Tsukada et al. 1986). Further work was undertaken by Avis et al. (1996) on lung cancer cell lines which, when subjected to various growth factors, increased their expression of 5-LOX and the generation of its products (5-HETE, 5-HPETE, LTB_4 and cys-LTs). Inhibitors of 5-LOX (AA861) and FLAP (MK886) impaired tumour cell growth (Avis et al. 1996) and the findings were repeated in breast cancer cell lines (Avis et al. 2001). Additionally they found that the administration of exogenous 5-HETE and cys-LT resulted in tumour cell proliferation of approximately 20-40% above control. Again FLAP inhibition resulted in reduced levels of 5-LOX and its metabolites, which was mirrored by a reduced expression of the apoptosis regulatory protein B-cell lymphoma 2 (Bcl-2) and an increase in the apoptosis promoter protein Bcl-2–associated X protein (Bax) as measured by immunocytochemistry and Western blotting. Of interest they demonstrated a concurrent elevation in other members of the LOX family, such as 15-LOX and its products including lipoxin A_4 raising the possibility of diversion down alternative eicosanoid pathways (Avis et al. 2001).

The effects of inhibition of the 5-LOX pathway on tumorigenesis have also been demonstrated *in vivo* (Rioux and Castonguay 1998). Mice were given a tobacco carcinogen along with aspirin, the 5-LOX inhibitor A79175, the FLAP inhibitor MK886, or a combination of both drugs. Mice fed a combination of aspirin and A79175 showed a reduction in lung tumour multiplicity of 87%, compared with mono-therapy with A79175 (75%), MK886 (52%), or aspirin (44%). In addition lung tumour volume reduced by 65% with A79175, 44% with MK886, and 35% with aspirin.

A number of investigators have demonstrated a role of the 5-LOX pathway in prostate cancer. Ghosh and Myers (1998) followed their initial observation that *n-6* fatty acids stimulate proliferation of prostate cancer cells *in vitro* through production of 5-HETE, by demonstrating that both AA861 and MK886 completely blocked 5-HETE production and induced massive apoptosis in a human prostate cancer cells *in vitro*. The cells were protected from apoptosis by the addition of exogenous 5-HETE. Gupta et al. then demonstrated that 5-LOX mRNA transcription was 6-fold greater and that of 5-HETE mRNA was 2.2-fold greater in malignant human prostatic tissue when compared with benign tissue (Gupta et al. 2001).

In pancreatic cancer tissue, immunohistochemical staining demonstrated expression of 5-LOX and BLT1, and reverse transcription PCR showed increased levels of *LTB4R* mRNA when compared with normal pancreatic tissue (Hennig et al. 2002). This group then subsequently showed that LTB_4 can stimulate proliferation and activation of the signalling molecules extracellular related kinases 1/2 in multiple human pancreatic cell lines (Tong et al. 2002).

A critical role for the *ALOX5* gene in leukaemia stem cells *in vivo* has recently been documented (Chen et al. 2009). They used a mouse model in which transplanted bone marrow cells treated with a retroviral vector of BCR-ABL fusion protein induces chronic myeloid leukaemia (CML). In mice with *ALOX5* gene deletion, BCR-ABL failed to induce CML, as they had depleted leukaemia stem cells (but not normal haematopoietic stem cells), with effects at the levels of differentiation, cell division, and long-term survival. Treatment with the 5-LOX inhibitor, zileuton, suppressed leukaemic stem cells in BCR-ABL-induced CML mice and prolonged their survival.

A role of the 5-LOX pathway in cell growth pathways in the gastrointestinal tract was shown in vitro by Ohd et al. (2000). The addition of LTB_4 and LTD_4 to intestinal cell lines led to an increase in expression and/or membrane accumulation of COX2, β-catenin, Bcl-2 and prostaglandin E_2 (PGE_2) production, in a time- and dose dependent manner. The associated reduction in cell death correlated well with LTB_4 and LTD_4 levels. The same group then investigated the expression of cysLT1R, along with other factors they had demonstrated to be up-regulated by LTD_4, in tissue arrays from colorectal adenocarcinoma specimens (Ohd et al. 2003). Increased expression of cysLT1R predominantly in the epithelial cells was shown in tumour tissue when compared with controls taken from the margins of the surgical specimens. Expression of 5-LOX, COX and the anti-apoptotic trans-membrane molecule, Bcl-XL (Bcl extra-large), but not Bax, was found to be elevated in tumour tissue that also demonstrated heightened levels of cysLT1R expression. Higher levels of cysLT1R expression were also associated with poorer survival. A further group from Japan used immunohistochemistry and Western blotting to demonstrate up-regulation of BLT1 receptors in human colon cancer cell lines (Ihara et al. 2007). Both inhibition of 5-LOX by AA861 or blockade of BLT1 receptors by the specific antagonist U-75302 suppressed cell survival and proliferation in a dose-dependent manner, which was attributable to an increase in apoptosis.

A summary of the 5-LOX molecular pathways implicated in carcinogenesis is shown in figure 1-F.

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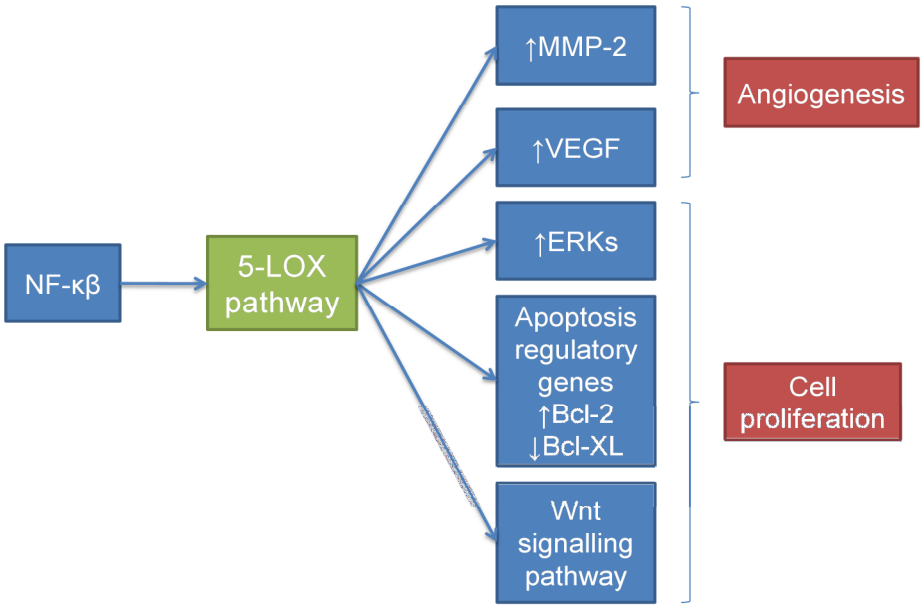


Figure 1-F: Possible role of the 5-LOX pathway in carcinogenesis demonstrated in published studies.

1.7.5 Evidence implicating the 5-lipoxygenase pathway in Barrett's adenocarcinoma

The first suggestion that LTs may play a role in Barrett's adenocarcinoma was made by Triadafilopoulos et al. who measured both PGE_2 and LTB_4 levels in normal squamous (control), reflux oesophagitis, and BM mucosal biopsy specimens taken at endoscopy (Triadafilopoulos et al. 1996). LTB_4, but not PGE_2, was significantly increased in both oesophagitis and BM biopsy specimens. In this study, treatment with the PPI omeprazole resulted in significant reductions of 30% in LTB_4 and 35% in PGE_2 levels. Chen et al. then reported first in 2002, with a subsequent paper in 2003 (Chen et al. 2002; Chen et al. 2003), showing that LTA_4H was over-expressed in rat OA compared with matched normal tissue samples, and that LTA_4H was expressed in infiltrating inflammatory cells and in the columnar cells in human OA. Bestatin, a LTA_4H inhibitor, reduced the incidence of OA by approximately 30% in a rat oesophageal carcinoma model. A further publication in 2004 reported on an increase in LTA_4H immunostaining intensity in BM, dysplasia and OA biopsies when compared with squamous controls, although the measurements used for analysis were open to observer bias (Chen et al. 2004). In an oesophago-gastro-duodenal anastamotic rat model of OA, they demonstrated that the 5-LOX inhibitor, zileuton, and the COX-2 selective inhibitor celecoxib, given either as mono-therapies or in combination, reduced the number of visible tumours (Chen et al. 2004). This corresponded with a significant reduction in the levels of both LTB_4 and PGE_2.

Hoque et al. (2005) showed immunohistochemical over-expression of 5-LOX in both squamous carcinoma and adenocarcinoma of the oesophagus, with no difference between histological types of cancer. In oesophageal cancer cell lines the 5-LOX inhibitor AA861 suppressed LTB_4 production and caused apoptosis. The BLT1 receptor antagonist U-75302 reversed the AA861-induced apoptosis, suggesting that BLT1 receptors mediate the action of endogenous LTB_4 on survival.

In summary, increased expression of proteins of the 5-LOX pathway is linked to cancer cell survival and proliferation in a number of tumour types. Furthermore, increased expression of this pathway occurs in BM and OA, and inhibition can result in reduced formation of OA *in vivo*. Important studies to date linking the 5-LOX pathway with cancer are summarised in table 1-A.

Table 1-A: Summary of important studies examining the role of the 5-lipoxygenase pathway in cancer.

Study (Year)	Tissue	Findings
Tsukada (1986)	Leukaemia cell lines	5-LOX inhibitors (Cirsiliol and AA861) showed potent anti-proliferative effects.
Avis (1996)	Small cell lung cell lines	5-LOX, FLAP, 5-HETE, LTB_4 expressed after exposure various growth factors. AA861 resulted in inhibition of cell line growth.
Ghosh (1998)	Prostate cancer cell lines	5-HETE expressed with massive apoptosis resultant from addition of FLAP inhibitor (MK886).
Anderson (1998)	Prostate cancer cell lines	5-LOX and FLAP expressed and inhibitors initiate cell death.
Rioux (1998)	Mice exposed to NNK (induces lung tumour formation)	Combination of aspirin and 5-LOX inhibitor (A-79175) most effective at reducing lung tumour multiplicity and incidence. LTB_4 increased and returned to that of controls on treatment inhibitors.
Ding (1999)	Human pancreatic cell lines	5-LOX (and 12-LOX) expressed (no expression in normal ductal pancreatic tissue). Inhibitors inhibited cell proliferation. 5-HETE, 12-HETE, AA and linoleic acid stimulated pancreatic cancer cell proliferation.
Ohd (2000)	Human intestinal cell line	LTB_4 and LTD_4 caused increase expression COX2, β catenin, bcl-2, PGE_2 and correlated with reduction in cell death
Sjolinder (2000)	Chronic myeloid leukaemia	Increased LTC_4S in neutrophils from patients with chronic myeloid leukaemia

Study (Year)	Tissue	Findings
Gupta (2001)	Human prostate benign and malignant tissue	*ALOX5* mRNA increased in malignant v benign (6-fold mRNA levels). 5-HETE increased.
Romano (2001)	Human malignant mesothelioma cell lines	5-LOX increased. AA861 inhibited malignant mesothelioma cell proliferation. 5LOX and 5-HETE upregulate VEGF.
Hennig (2002)	Human pancreatic cell lines	Increase 5-LOX and LTB_4 (versus normal pancreatic tissue)
Chen (2002)	Rat surgical OA model	Increase 5-LOX and 5-HETE (and 8-, 12-, 15-HETE) compared to proximal normal oesophagus. The NSAID (Sulindac) + nordihydroguaiaretic acid (NDGA) reduced incidence of tumour. NDGA alone reduced but not statistically significant.
Chen (2003)	Rat and human OA	Increased LTA_4H expression (versus adjacent normal tissue). Localised to inflammatory cells and columnar in human tissue. Intraperitoneal LTA_4H inhibitor (Bestatin) reduced incidence of OA in rat model.
Ohd (2003)	Tissue arrays from colorectal cancer samples	Increase in cysLT1, 5-LOX (and COX-2). Expression cysLT1R correlated with poorer survival in Duke's B patients.
Chen (2004)	Rat and human OA	Progressive increase 5-LOX through oesophagitis to Barrett's to OA. High dose 5-LOX inhibitor (Zileuton) and COX-2 inhibitor (Celecoxib) reduced LTB_4 levels and incidence of tumour (dose dependent) in rat surgical model

Study (Year)	Tissue	Findings
Ye (2004)	Mice exposed to dextran sulphate sodium (induces colonic inflammation) and cigarette smoke	Cigarette smoke increased 5-LOX expression in inflammation associated colonic adenomas. Associated upregulation of MMP-2 and VEGF. 5-LOX inhibition (AA861) reduced incidence of adenoma formation and reduced angiogenesis with associated reduction in MMP-2 activity and VEGF expression.
Hoque (2005)	Human oesophageal squamous and OA	5-LOX increased (versus normal) AA861 suppressed LTB_4 production. LTB_4R antagonist (U-7530) blocked AA861 induced apoptosis.
Hennig (2005)	Mice pancreatic cancer	LTB_4R antagonist (LY293111) reduced tumour growth and incidence of metastasis. Effect most marked when combined with Gemcitabine.
Cianchi (2006)	Colon cancer cell lines	Increased expression COX2 and 5-LOX. Single inhibition increased opposite pathway activation. Dual inhibition prevented this and resulted in apoptosis, prevention tumour cell proliferation, decrease Bcl-2, increase Bax.
Ihara (2007)	Human colon cancer	Increase BLT1 expression (versus normal) including adenocarcinoma cells. AA861 and LTB_4 receptor antagonist (U75302) had negative effect on cell survival.
Schroeder (2007)	Pre- and malignant lung cell lines	COX2, MK886, and cysLTR antagonist (REV5901) more potent than single or dual inhibition. Sensitivity correlated with production of metabolites (not 5-LOX or FLAP). Partial protection afforded when PGE_2 or 5HETE added.
Tong (2007)	Pancreatic cell line	LTB_4 receptor antagonist (LY293111) induced apoptosis and S-phase cell cycle arrest with downregulation of CDK2, cyclin A and cyclin E.

Study (Year)	Tissue	Findings
Hennig (2008)	Human pancreatic tissue	BLT2 overexpressed in malignant intraductal papillary mucinous neoplasia and adenocarcinoma tissue. Treatment of pancreatic cancer cell lines with selective BLT2 agonist resulted in tumour cell proliferation.
Funao (2008)	Human renal cell cancer and cell lines	CysLT1R overexpressed and receptor antagonist induced apoptosis.
Rocconi (2008)	Human ovarian cancer tissue	5-LOX, BLT1 and 2 expressed. BLT2 expression correlated with advanced disease.
Melstrom (2008)	Colonic adenomatous polyps and cancer + colon cancer mouse model	Increase in 5-LOX expression (versus normal colonic tissue). Inhibition in 5-LOX resulted in reduction in tumour growth.
Sveinbjörnsson (2008)	Human neuroblastoma tissue and cell lines	Overexpression of 5-LOX, FLAP, LTA_4H, and LTC_4S. Inhibition of 5-LOX (AA-861), FLAP (MK-886) and Cys-LT1R (montelukast) inhibited cell growth through induction of G(1)-cell cycle arrest and apoptosis.
Chen (2009)	Mouse BMT model of CML	Absence *Alox5* led to failure of chronic myeloid leukaemia development and impairment function leukaemia stem cells (LCS). Treatment with zileuton impaired LCS and prolonged survival.
Matsuyama (2009)	Human testicular cancer	CysLT1R expressed strongly in testicular cancer cells. Receptor antagonist inhibited cell growth through early apoptosis.
Wasilewicz (2010)	Colonic adenomas	5-LOX expression correlated with risk factors for malignant transformation (size, high grade dysplasia)
Fischer (2010)	Various tumour cell lines	Certain 5-LOX (AA861, MK-886, REV-5901) inhibitor resulted in cytotoxic and antiproliferative effects independent of 5-LOX activity.

Study (Year)	Tissue	Findings
Wang (2011)	Glioma stem-like cells	Inhibition of 5-LOX by dl-nordihydroguaiaretic induced differentiation in glioma stem-like cells.
Amirian (2011)	Prostate cancer	Single nucleotide polypmorphism in *LTA4H* (rs1978331) was inversely associated with prostate cancer risk.
Sarveswaran (2011)	Prostate cancer cell lines	5-LOX inhibition-induced apoptosis occurs via inactivation of protein kinase C-epsilon.
Mohammed (2011)	Mouse model for familial adenomatous polyposis ($APC^{min/+}$ mice)	The dual 5-LOX/COX-2 inhibitor licofelone suppressed small intestinal and colonic tumour formation in $APC^{min/+}$ mice.
Park (2012)	Head and neck squamous cell cancer cell lines	Inhibition of COX-2 increased LTB_4 but showed little growth inhibitory effects. Combined COX-2 and 5-LOX inhibition resulted in decrease in cell proliferation dependent on 5-LOX expression in cells.
Zhao (2012)	Hepatoma cell lines	LTB_4 activated NF-κβ in a dose-dependent manner.
Kummer (2012)	Papillary thyroid cancer cell lines	Transfection with *ALOX5* increased MMP-9 secretion and invasion across the extracellular matrix barrier. 5-S-HETE increased MMP-9 in a dose-dependent manner. 5-LOX and MMP-9 inhibitors reversed *ALOX5* enhanced invasion.

1.8 Current management strategies for high grade dysplasia in Barrett's oesophagus

1.8.1 Current practice

Difficulties with the management of HGD in BM derive from problems inherent with the endoscopic diagnosis of the disease. Barrett's segments are heterogeneous and diagnosis by endoscopic biopsy can sample only a fraction of the whole segment. Concerns arise around the risk of missing invasive carcinoma, despite biopsies demonstrating HGD only. A number of retrospective studies have demonstrated the presence of metachronous invasive cancer in up to 40% of those with an initial pre-operative diagnosis of HGD (Edwards et al. 1996; Heitmiller et al. 1996). Based on these arguments, the British Society of Gastroenterologists issued guidance in 2005 suggesting that surgical oesophagectomy should be the first line therapeutic option for HGD in those fit for the procedure (Watson et al. 2005). However, surgical oesophagectomy remains a high risk procedure, even in tertiary centres, with modern operative mortality of approximately 3.2%, and post-operative complications in up to 40% (Palser et al. 2009). Data also suggests that even 3-years after oesophagectomy, patients suffer significant morbidity (Lagergren et al. 2007). The adoption of a first line surgical strategy has therefore been called into question.

1.8.2 Alternative therapies

Given the drawbacks of oesophagectomy as a first line treatment strategy, a variety of alternative endoscopic options for the management of HGD in BM have been tried (see Table 1-B). Thermal ablation involves using either multi-polar electro-coagulation (MPEC), argon plasma coagulation (APC), or radiofrequency ablation (RFA) to induce very high tissue temperatures and subsequent cell death. Although MPEC has been utilised widely in non-dysplastic Barrett's, only APC and RFA have been used with any success in dysplastic Barrett's. The role of RFA in Barrett's is discussed in detail further on in this section.

1.8.2.1 Argon Plasma coagulation

APC uses high-frequency, monopolar current of ionized argon gas resulting in coagulation of the epithelium. Only three small observational studies have reported use with APC in HGD (Morris et al. 2001; Van Laethem et al. 2001; Attwood et al. 2003). Although eradication of dysplasia was achieved in up to 100%, perforation occurred in up to 4%, and in one case series, one (out of 7) progressed to OA (Van Laethem et al. 2001).

1.8.2.2 Laser therapy

Two types of lasers have been used in dysplastic Barrett's: neodymium-yttrium aluminium garnet (Nd-YAG) and potassium titanyl phosphate (KTP). Both use an intense beam of light to produce injury to the target epithelium. Experience is limited to the treatment of only 18 patients with HGD. Eradication of dysplasia was variable, and in one mixed case series (5 with HGD, 16 LGD, and 10 non-dysplastic Barrett's) one out of 5 with HGD at baseline progressed to OA requiring oesophagectomy (Fisher et al. 2003).

1.8.2.3 Endoscopic mucosal resection

Endoscopic mucosal resection (EMR) of the entire affected mucosa has been explored. It has the added advantage of allowing histological assessment of the entire Barrett's segment, with the potential of a more sustained treatment response. None have progressed to OA thus far in any of the studies (Ell et al. 2000; Seewald et al. 2003; Peters et al. 2006; Soehendra et al. 2006; Lopes et al. 2007; Pech et al. 2008). High technical expertise is nevertheless required to accomplish the technique with a low complication rate. Oesophageal perforation may occur in up to 5% and bleeding in up to 25%. Strictures are mainly described in those undergoing EMR of greater than 75% of the circumference of the oesophagus, and in this situation may occur in up to 70% of cases (Soehendra et al. 2006; Pech et al. 2008).

1.8.2.4 Cryotherapy

Cryotherapy is a promising evolving technique that involves applying liquid nitrogen directly onto the mucosa using a spray catheter introduced through the working channel of the endoscope. The tissue is frozen for approximately 40 seconds either in two cycles of 20 seconds or in four cycles of 10 seconds. Results from a retrospective cohort study demonstrated that of 60 patients with HGD who completed the cryotherapy sessions, 58 had complete eradication of HGD (97%), 52 (87%) had complete eradication of all dysplasia, and 34 (57%) had complete eradication of intestinal metaplasia (Shaheen et al. 2010). Complications included chest pain (2) and strictures (3), but no perforations. These early results would appear to suggest cryotherapy is a highly effective and safe technique. It was however a non-randomised, retrospective study without a control group and with only limited (10.5 month) follow up. Further data are required before cryotherapy can be envisaged as an alternative ablative technique.

1.8.2.5 Photodynamic therapy

Ablation of the Barrett's segment with photodynamic therapy (PDT) involves intravenous administration of a photosensitising agent, either intravenous sodium

porfimer, oral 5-aminolaevulinic acid (5-ALA), or meta-tetrahydroxyphenyl chlorin (mTHPC), followed by endoscopic light laser exposure to the Barrett's segment. This results in the generation of oxygen radicals in the photosensitised tissues which leads to cell death. A large randomised multicentre multinational trial has demonstrated that treatment with sodium porfimer based PDT is superior to omeprazole alone at eliminating HGD (77% vs 39%, $p<0.0001$), and additionally in preventing progression to invasive cancer (15% v 29%, $p=0.027$) (Overholt et al. 2007). Experience with mTHPC PDT is limited to a case series of less than 10 patients (Etienne et al. 2004; Lovat et al. 2005). 5-ALA PDT is associated with fewer side-effects and success rates of up to 100%, but experience is limited to single centre case series, with no RCT to date (May et al. 2002; Pech et al. 2005). Three prior cost-effective analyses have demonstrated PDT's superiority when compared with oesophagectomy or surveillance only (Hur et al. 2003; Shaheen et al. 2004; Vij et al. 2004). However, the widespread acceptance of PDT has been held back by treatment-related adverse events in up to 94% of patients and by a high incidence of oesophageal stricturing in 39% with sodium porfimer based PDT (Overholt et al. 2005). Furthermore, it has been associated with the presence of 'buried' foci of residual Barrett's tissue (sub-squamous metaplasia), which are associated with the emergence of OA at a later date (Fitzgerald 2003; Bergman and Fockens 2006; Overholt et al. 2007). As such, PDT is only undertaken by a limited number of centres, and is not currently recommended as first line treatment in those fit for surgery (Watson et al. 2005).

Table 1-B: Summary of outcomes and complications of endoscopic techniques used to eradicate HGD in Barrett's oesophagus

Modality (Author, Year)	Study design	Eradication of dysplasia	Stricture rate	Perforation	Other
PDT (Overholt, 2005; Overholt, 2007)	RCT	77-97%	Up to 36%	<1%	69% photo-sensitivity
EMR (Ell, 2000; Seewald 2003; Peters, 2006; Soehendra, 2006; Lopes, 2007; Pech, 2008)	Case series	75-100%	14-70%	Up to 5%	Bleeding 20%
APC (Morris, 2001; Van Laethem, 2001; Attwood, 2003)	Case series	86-100%	0-9%	Up to 4%	Chest pain 2%
Laser (Nd-YAG or KTP) (Gossner, 1999; Weston, 2002; Fisher, 2003)	Case series	62-100%	0-12%	3%	Bleeding 6%
Cryotherapy (Shaheen, 2010)	Case series	87%	3%	0	Chest pain 2%

1.8.3 Radiofrequency ablation

1.8.3.1 The Technique

RFA utilises a high frequency alternating current and generates tissue injury by converting electrical energy into heat. Delivery of continuous, un-modulated, sine wave alternating current results in heat-induced coagulation necrosis. The Bârrx HALO device is an endoscopic delivered, novel, balloon-based therapy capable of delivery of radiofrequency energy to the oesophagus in a controlled fashion. It consists of an energy generator and then two different devices (Figure 1-G). The HALO^{360} is a catheter passed by endoscopic wire guidance and can deliver a pre-set amount of radiofrequency energy density to a 360˚ ablation field. On the surface of the balloon is a 3-cm-long bipolar microelectrode consisting of 60 tightly spaced electrode rings. The electrode rings each completely encircle the balloon and alternate in polarity. Each ring is approximately 250 µm wide and is approximately 250 µm from their neighbouring electrode. The HALO^{90} ablation catheter attaches to the end of the endoscope and has a smaller electrode surface area, approximately 20 mm long and 13 mm wide, and covers an approximate 90˚ radius. This allows a more focal selective ablation of residual Barrett's tissue. The electrode array is identical in pattern to the 360º.

Figure 1-G: The Bârrx HALO system
(a) Energy generator (b) 360º ablation catheter (c) 90º ablation catheter

The procedure is undertaken as a day case procedure under conscious sedation. After assessment of the area to be treated, the inner diameter of the oesophagus is first sized using a separate inflatable sizing balloon, and utilising an automatic pressure: volume algorithm. An ablation catheter with a size commensurate with the measured internal diameter is then passed over a guide wire, and ablation delivered, starting proximally, by inflation of the balloon and then delivery of energy by the energy generator. The catheter is then realigned with the distal edge of the ablation zone (with a slight overlap), and further ablation applied. This process is then repeated until

complete ablation of the Barrett's segment, with the ablation zone extending to the top of the gastric folds (Figure 1-H).

Figure 1-H: Illustration of ablation of a Barrett's segment using the 360º device

1.8.3.2 Initial dosimetry studies

Initial studies evaluated the endoscopic and histological effects of circumferential RFA in porcine models as well as in the normal squamous portion of the human oesophagus in patients undergoing oesophagectomy for OA (Ganz et al. 2004). In this multi-phase study, the authors demonstrated that complete circumferential ablation of porcine and human oesophageal epithelium could be achieved without injury to the sub-mucosa. Ablation depth was found to be linearly related to the energy density delivered. This led to further dosimetry studies to assess the optimal energy density and treatment parameters to achieve complete ablation (Dunkin et al. 2006). In this study, patients underwent circumferential ablation of a 3cm section of squamous oesophageal epithelium just prior to oesophagectomy. They found 12 joules/cm^2 (J/cm^2) was the optimum energy density, with lower densities resulting in only partial ablation. Mean residual tissue depth was 35 µm, with no burn deeper than the muscularis mucosa. The final dosimetry study was conducted on patients just prior to oesophagectomy on areas with HGD in Barrett's oesophagus undergoing two, three or four applications of circumferential ablation at 10, 12, or 14J/cm^2 (Smith et al. 2007). Complete ablation of intestinal metaplasia and HGD was achieved in nine out of ten ablation zones (at 12J/cm^2), with one failure attributed to incomplete overlap. Again ablation depth was dose related, and limited to the muscularis mucosa.

1.8.3.3 Studies evaluating radiofrequency ablation in Barrett's metaplasia

Studies were then undertaken in non-oesophagectomy patients with non-dysplastic Barrett's oesophagus. The Ablation of Intestinal Metaplasia trial (AIM-trial) included patients with non-dysplastic Barrett's and was conducted in two phases; a dosimetry (AIM-I) and effectiveness (AIM-II) phase (Sharma et al. 2007). The initial dosimetry phase demonstrated 10J/cm^2 or 12J/cm^2 were superior to lower energy levels, without any serious adverse events. 10J/cm^2 (x2) was selected for the effectiveness phase. Twenty-four adverse events were reported in 16 patients: transient nausea (8), mucosal injury or scarring (2), chest pain or throat pain (9), fever (2), mild bleeding which stopped spontaneously (1), and sedation related events (2). At 12-month follow up complete remission of intestinal metaplasia was achieved in 48 of 69 patients (70%).

Further published articles have reported on the longer term follow-up of the AIM-II cohort (Fleischer et al. 2008). In the study by Fleischer *et al*., further circumferential ablation therapy was performed at 4 months, followed by focal ablation of residual segments on up to 3 occasions as necessary. Complete response to intestinal metaplasia was seen in 60 of 61 patients (98%) at 30 month follow up. There were no strictures, no serious adverse events, and no buried glandular mucosa. Five-year follow up data have now been published. Of the 50 patients they contacted, 46 had complete response to intestinal metaplasia (92%). Of the four with residual Barrett's, they demonstrated that re-establishment of complete response was achievable by delivering one further treatment session with focal ablation at 12J/cm^2 (Fleischer et al. 2010).

1.8.3.4 Studies evaluating radiofrequency ablation in dysplastic Barrett's

The role of RFA in LGD has been evaluated in two studies. In a case series of 39 patients with LGD treated with circumferential and focal ablation, complete eradication of dysplasia was achieved in 95%, and complete eradication of IM in 87% at 2-year follow up (Sharma et al. 2009). The only published RCT on RFA included 64 patients with LGD, of whom 42 were assigned RFA and 22 to a sham procedure (Shaheen et al. 2009). Complete eradication of LGD was achieved in 90% in the RFA group (v 23% sham, $p<0.001$), with complete eradication of IM in 81% (v 4% sham, $p<0.001$). Two patients did progress to HGD during the trial period.

Initial studies included only very small numbers of patients with HGD (Roorda et al. 2007; Hernandez et al. 2008). Subsequent case series suggested that eradication of HGD could be achieved in 90.2% (Ganz et al. 2008) and 98% (Pouw et al. 2008). The most robust data on the role of RFA in HGD has come from a multicentre sham-controlled RCT in the US* (Shaheen et al. 2009). Sixty-three patients with HGD were included, of whom 42 were assigned RFA and 21 to a sham procedure. Complete eradication of dysplasia was achieved in 81% on an intention to treat protocol at 12-month follow up. One out of the 42 patients with HGD (2%) (versus 4/21 – 19% in the sham group) progressed to cancer during the trial period. The disease was intra-mucosal cancer which, although not stated, could be removed endoscopically without need for oesophagectomy. The authors measured chest pain using a visual analogue score (VAS, scale 0-100). On day one, mean VAS was 22 (versus zero in the sham group), dropping to zero by day eight. Serious adverse events were infrequent, with one upper gastrointestinal haemorrhage treated endoscopically (patient was taking anti-platelets), and two episodes of chest pain requiring overnight admission. The

* This trial included patients with both LGD and HGD. The results of those with LGD are discussed above

stricture rate was 6%, all of which resolved with oesophageal dilatation (average of 2.6 sessions required).

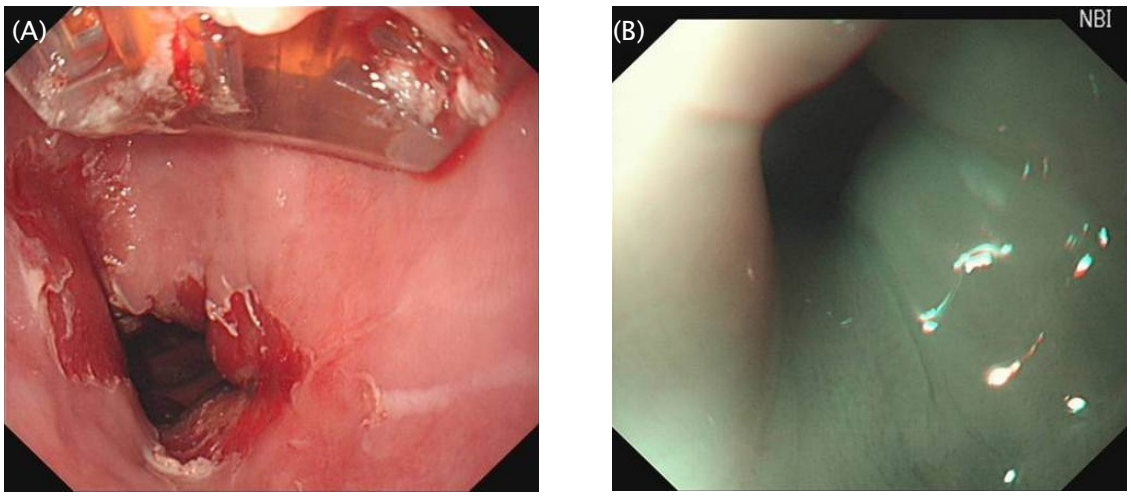


Figure 1-I: Treatment of Barrett's metaplasia by the HALO 90 device
(A) During treatment and (B) appearance of the oesophagus under narrow band imaging (NBI) three months later. The lining of the oesophagus has reverted to squamous mucosa.

1.8.4 Current guidance from the National Institute for Health and Clinical Excellence (NICE)

NICE issued initial guidance in December 2007 on the use of circumferential RFA for the ablation of Barrett's oesophagus. The conclusion of the guideline was that evidence was insufficient to recommend the use of circumferential RFA in non-dysplastic Barrett's oesophagus, and thus stated that the procedure should only be used in the context of clinical research. Of note this guidance only dealt with the issue of non-dysplastic Barrett's; a different subset of patients with a much lower risk of developing OA (estimated rate of conversion to cancer of 0.5% per annum). Additionally, the HALO system now utilises two complementary delivery methods to achieve ablation of the affected tissue (the HALO^{360} and HALO^{90}). Recent experience appears to demonstrate that combining the two techniques results in improved outcome (Gondrie et al. 2008a), although this has not been subjected to a randomised clinical trial.
Since the development of the model in this thesis, NICE have issued further guidance (NICE 2010a). This is included in the discussion chapter.

1.8.5 Current economic studies evaluating the role of RFA in Barrett's oesophagus

At the time of this work, no economic studies had been published looking at the cost-effectiveness of RFA in the treatment of HGD. A subsequent study from the US has been published (Inadomi et al. 2009), and this is discussed later. The economic models

evaluating ablative studies in the treatment of Barrett's and HGD, and their relevance to the construction of the model in this study are discussed in the methods section.

1.8.6 Current economic cost of the problem in the UK

The true incidence of HGD in the UK is not currently known, but it is estimated that between 0.5-2% of the adult population in the Western world have Barrett's oesophagus (Corder et al. 1996; Csendes et al. 2000; Ronkainen et al. 2005). The annual incidence of HGD in those undergoing surveillance is 0.9% per annum (Sharma et al. 2006). Based on the 2001 census and a screening pick up rate of 30-35% per annum one would expect 2000-3000 cases HGD in the UK per year. If all were to undergo oesophagectomy as per current guidance, one would expect a cost to the NHS of £15-20 million per year.

1.9 Summary and Aims of thesis

1.9.1 The role of leukotrienes in the pathogenesis of Barrett's adenocarcinoma

A mounting body of evidence suggests a key role for LTs in promoting cancer cell growth, possibly by suppression of apoptosis and promotion of neo-angiogenesis. Their precise roles and cellular sources are nevertheless poorly defined. Preliminary work by our group has established by enzyme immunoassay that tissue levels of LTB_4 and LTC_4 are significantly increased in OA tissue, following *ex vivo* stimulation of cultured biopsies from patients with BM and OA. This study aims to relate the generation of these lipid products to the expression of 5-LOX pathway proteins within the biopsies.

1.9.1.1 Hypothesis

5-LOX pathway activity is up-regulated progressively through metaplasia to adenocarcinoma, by induction of enzyme expression and/or infiltration by inflammatory cell-types.

1.9.1.2 Aims

To facilitate a greater understanding of the cellular sources of eicosanoid products implicated in the molecular pathogenesis of BM and OA, immunohistochemistry and laser microdissection will be utilised to localise and quantify 5-LOX pathway expression.

1.9.2 Cost-utility analysis of radiofrequency ablation or oesophagectomy for the management of high-grade dysplasia in Barrett's oesophagus

Options for the treatment of HGD in BM are imperfect. Current guidance recommends oesophagectomy, which is associated with high morbidity and mortality. RFA is a promising new technology, with early trial results demonstrating its efficacy in the treatment of HGD in BM, with minimum morbidity and mortality. The cost per patient to the NHS is not currently known and there has been no RCT comparing RFA and oesophagectomy.

The cost-effectiveness of introducing RFA for the treatment of HGD in the UK is unknown. It is this group of patients, with the highest risk of development of OA and currently such limited treatment options that are likely to be the initial target group for therapy with RFA. Before introducing such a treatment into the UK, it is important to evaluate the lifetime benefits and costs of RFA compared with the standard current practice of oesophagectomy. This will help bring clarity to the difficult clinical situation of selecting the most appropriate strategy in managing patients with HGD in BM.

Health economic evaluation using a cost utility analysis allows us to consider the costs and, using utility as a measure of health state preference, estimate quality-adjusted life years, prior to the introduction of a new technology into a health care setting. This is especially relevant to the appropriate allocation of health care expenditure within a limited budget. In addition, when uncertainty exists, it allows us to interrogate the available data and form conclusions about where further study might be needed.

1.9.2.1 Hypothesis

Radiofrequency ablation is a cost-effective option for treatment of high-grade dysplasia in Barrett's in a UK setting.

1.9.2.2 Aim

To model whether RFA followed by oesophagectomy for persistence or recurrence of HGD is a cost-effective option for the treatment of BM with HGD when compared with the current UK recommendation of oesophagectomy, and to identify critical parameters to guide further research.

CHAPTER 2: Methodology of laboratory studies

This chapter outlines the methodology used for the laboratory studies evaluating expression and localisation of the 5-lipoxygenase (5-LOX) pathway in Barrett's metaplasia (BM) and oesophageal adenocarcinoma (OA). Patient recruitment, sample collection, ethical approval and choice of control tissue are discussed in section 2.1. The protocol for immunohistochemical analysis of 5-LOX pathway proteins in the sample tissue is outlined in section 2.2. The devised protocol for the laser capture of cellular areas from the tissues for mRNA analysis is presented in section 2.3. Section 2.4 concerns itself with statistical considerations for data handling.

2.1 Patient recruitment

2.1.1 Patient selection and biopsy collection

Ethical approval for the study was granted by Isle of Wight, Portsmouth and Southeast Hampshire Research Ethics Committee (REC reference number: 08–H0501–3) (see appendix A). All patients gave informed consent, after both a written explanation (provided at least 24 hours in advance of signing consent) and a verbal explanation of the study to be undertaken (see appendix B).

Patients were included in the study if they had a diagnosis of:

i) BM with a segment length of at least 3 cm

ii) OA

44 patients were recruited in total; 31 with Barrett's oesophagus, 13 with a prior diagnosis of oesophageal cancer. Of these 6 with oesophageal cancer were excluded due to the finding of junctional tumours (3), undifferentiated carcinoma (1), and lack of OA tissue in the sample taken (2). The individual patient characteristics can be found in the results section for the immunohistochemistry study (section 4.4) and gene expression analysis (section 5.3.3).

Fresh and adjacent formalin-fixed oesophageal biopsies were then obtained at upper gastrointestinal endoscopy. Biopsies from those with BM were taken 2cm proximal to the top of the gastric folds. Length of Barrett's segment and current use of proton pump inhibitors (PPI) and aspirin were recorded. Adjacent biopsies were taken and submitted for formalin fixation, processed using routine methods into haematoxylin and eosin-stained sections and assessed by a histopathologist expert in the field of

gastrointestinal pathology, blinded to any study results. BM was defined as the visualisation of metaplastic columnar epithelium with the histological finding of intestinal metaplasia.

2.1.2 Choice of control tissue

The normal lining of the oesophagus is with squamous tissue, and this therefore is the starting point in the progression to BM and then OA. Therefore from a subset of patients, samples were taken from of squamous tissue from an area just proximal to the diseased oesophagus.

2.2 Immunohistochemistry

2.2.1 Introduction

Immunohistochemistry (IHC) allows the detection of specific antigens in cells within tissue sections by the use of antibodies specific to the antigen of interest. Advantages of the technique include the ability to directly visualise the location of positively stained cells within the tissue section. This technique was utilised to examine expression within BM and OA biopsies of the four main enzymatic proteins of the 5-LOX pathway: 5-LOX, FLAP, LTA_4H, and LTC_4S. The aim was to correlate the expression of these proteins with tissue staining of white cell subsets, namely neutrophils, macrophages/monocytes, eosinophils, and lymphocytes,

2.2.2 Fixing media for immunohistochemical analysis

Glycol methacrylate is a hard resin which can be utilised in biopsy fixation and allows cutting of ultra-fine 2µm sections, whilst preserving tissue morphology and preserving antigens for immunohistochemistry.

2.2.3 Biopsy processing

The fresh biopsy specimens were immediately fixed in ice-cold acetone containing the protease inhibitors phenylmethylsulphonylfluoride (PMSF, 2mM) and iodoacetamide (20mM) (Sigma, Poole, UK) for 16-24h at -20ºC, then embedded in glycol methacrylate resin (Park Scientific Ltd, Northampton, UK) as previously described (Britten et al. 1993). The biopsies were then stored in secure -20^0C freezers until required.

2.2.4 Biopsy quality assessment

Biopsies can be of varying quality and thus were assessed individually before IHC staining was undertaken. The resin blocks containing each biopsy were trimmed and filed to size and then, using the microtome, 2µm sections were cut and stained with toluidine blue. The biopsy was assessed under the light microscope for tissue type,

epithelial quality, quality of stromal area, and overall quality. If the section seen was of poor standard then further sections were taken from deeper within the biopsy and reassessed as above. Thus a level in the biopsy was found which was deemed to be of adequate histological standard. If no such level was found then the biopsy was excluded from further immunohistochemical analysis.

2.2.5 Primary Antibody titration

Optimum antibody titre was determined by serial dilutions using human tonsillar tissue, known to express all of the antigens of interest. Titrations started at a 1:50 dilution and were progressively titrated out until disappearance of positive staining. Slides were then assessed by light microscopy and number of positive staining cells per high powered field (x 40 magnification) was recorded, in 6 separate random fields. A titration curve was plotted to demonstrate the antibody titre prior to a reduction in the number of positive cells. Optimum antibody titre was then defined as the dilution of antibody resulting in the maximum specific staining (i.e. highest cell count) but with the least amount of background under conditions in the used laboratory (Figure 2-A).

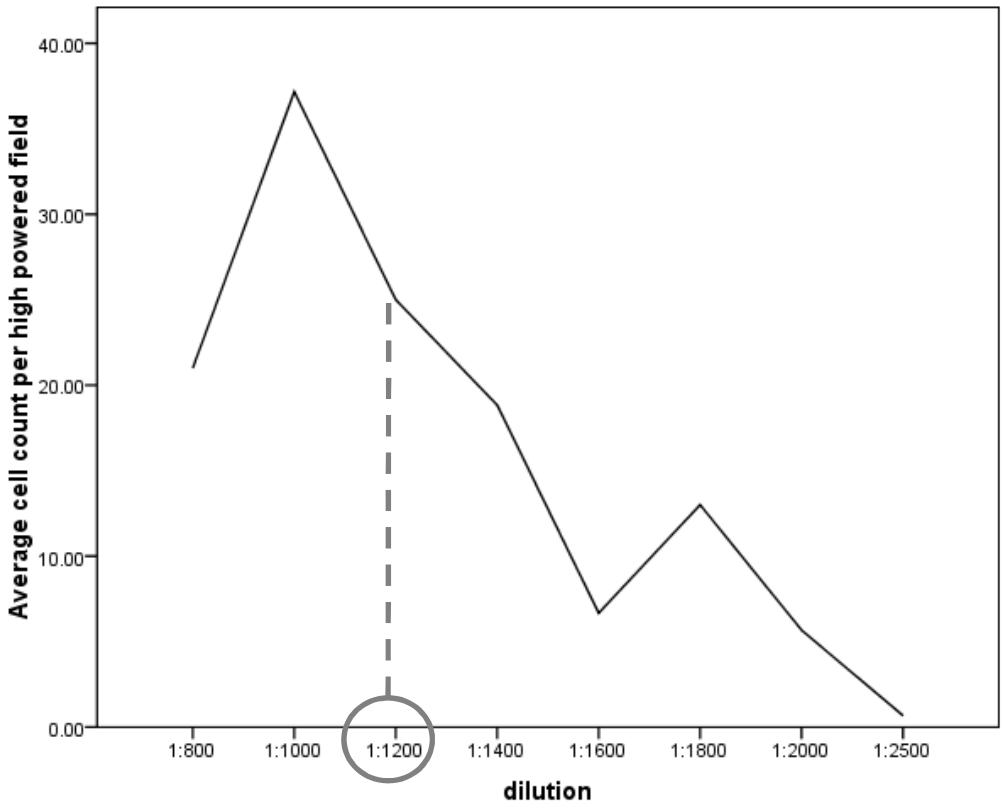


Figure 2-A: Example of titration curve used to calculate the optimum antibody dilution. Here FLAP is shown, and although 1:1000 titre resulted in most number of positive cells, a 1:1200 titre was chosen as a compromise to minimise the impact of background staining.

The following primary antibodies were used at the following dilutions:

Antibody	Source	Host	Class	Optimal dilution µg/ml
5-Lipoxygenase (5-LOX)	Cayman Chemical Co. (Tallinn, Estonia)	Rabbit	IgG	2
5-Lipoxygenase Activating Protein (FLAP)	Kind gift from Dr John Menke, Merck & Co. Inc.	Rabbit	Uncertain	1:1200 titre used*
Leukotriene A_4 Hydrolase	Kind gift from Dr John Menke, Merck & Co. Inc.	Rabbit	Uncertain	1:2500 titre used*
Leukotriene C_4 Synthase	Kind gift from Dr John Menke, Merck & Co. Inc.	Rabbit	Uncertain	1:1800 titre used*
Pan-cytokeratin	Dako	Mouse	IgG1	0.34
Neutrophil elastase (NE)	Dako	Mouse	IgG1	0.07
CD68	Dako	Mouse	IgG3kappa	0.2
CD3	Dako	Mouse	IgG1	1
EG2	Diagnostics Development	Mouse	IgG1	0.8

*Concentration of original stock unknown

2.2.6 Immunohistochemistry

All steps were carried out at room temperature unless mentioned.
Firstly 2µm tissue sections were cut, floated out on ammonia water (1ml ammonia in 500ml distilled water), and picked up on glass slides coated with poly-L-lysine, which enhances tissue adhesion to the slide. Two sections were placed on each slide. The slides were air dried for at least 60 minutes and then either stored for a maximum of 2 weeks at -20^{0}C, or used immediately.

Slides were laid in staining trays and then treated with the membrane solubiliser Tween-20 wash. Endogenous peroxidases were then inhibited by the application of 0.1% sodium azide containing 1/100th volume of 0.3% hydrogen peroxide and incubated at room temperature for 30 minutes. The reaction was stopped by three Tris-buffered saline (TBS) washes of five minutes each.

2.2.6.1 Blocking steps

The avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK) was used to block endogenous biotin, biotin receptors and avidin binding sites (Cowburn et al. 1998). Avidin block was applied for 20 minutes, washed briefly three times with TBS for 2 minutes per wash. Biotin block was then added and left for a further 20 minutes, followed by a repeat brief wash, and then culture medium was added for 30 minutes. The culture medium was removed by gentle drainage and the primary antibody added to each section at the appropriate titre. Cover-slips were used to avoid drying out of the section and even antibody distribution. Slides were incubated overnight at room temperature with monoclonal primary antibody or at 4^0C with polyclonal primary antibody.

2.2.6.2 Avidin-Biotin Complex method

In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex, as shown in Figure 2-B (Hsu et al. 1981).

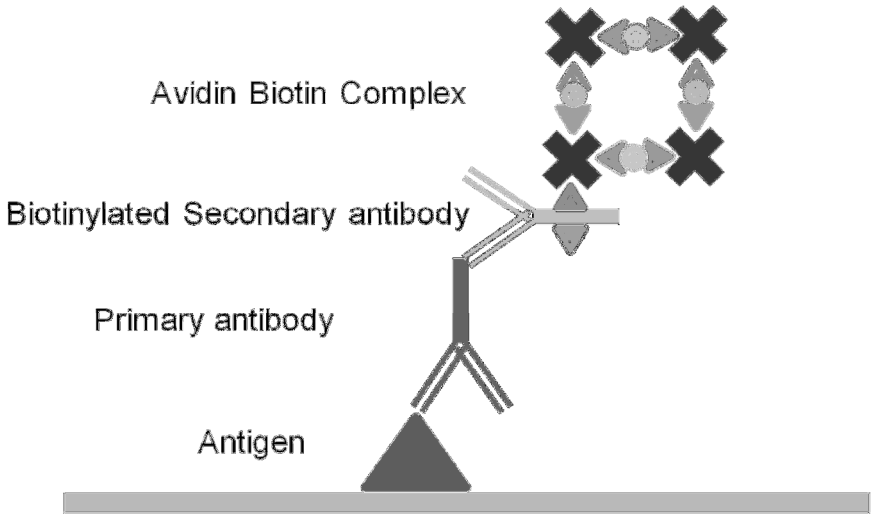


Figure 2-B: Avidin Biotin Complex (ABC) method

If slides were incubated at 4^0C they were allowed to equilibrate to room temperature for 30-60 minutes prior to removal of the primary antibody by washing in TBS for five minutes three times. The second stage biotinylated antibodies were then added at the following concentrations, and incubated for 2 hours:

Secondary biotinylated antibody	Source	Host	Optimal dilution (µg/l)
Anti-rabbit	Dako	Swine	0.81µg/ml
Anti-mouse	Dako	Rabbit	0.77ug/ml

The slides were drained and washed in TBS for five minutes three times. The ABC was made up at least 30 minutes prior to adding to slides by diluting both avidin and biotin at a 1:75 dilution in TBS and then mixing by gentle inversion. The ABC was then added to the sections and incubated for 2 hours. The slides were drained and washed in TBS for five minutes three times prior to addition of the chromagen.

2.2.6.3 Choice of chromagen

Chromagen's are substances which upon being oxidized become coloured insoluble products, and thus allow visualisation of the primary antibody. The choice of chromagen depends upon the type of quantification of staining required. 3, 3'-diaminobenzidinetrahydrochloride (DAB) produces a brown end product which varies in staining intensity dependent on the degree of oxidation. It is thus suitable for measuring staining intensity. 3-amino-9-ethylcarbazole (AEC) produces a rose-red end product, which when used with Mayer's haematoxylin, provides valuable contrast with the dark purple staining nuclei, and thus is a good choice for assessing the number of cells expressing the antigen of interest. AEC was chosen for the majority of the samples when assessment by cell counting was anticipated. DAB was used to look at specific binding on the epithelium by assessment of intensity of staining.

AEC was applied for 20 minutes and DAB for 10 minutes, prior to washing with TBS and then running tap water for five minutes. A counter-stain Mayer's haematoxylin was used. This was a water based stain to prevent solubilisation of AEC which is alcohol soluble. The slides were immersed for one and half minutes in this counter-stain, followed by further washing in running tap water. Cover slips were applied with an aqueous mount and allowed to dry prior to analysis.

2.2.7 Negative controls

Primary antibody specificity is of utmost importance in immunohistochemistry, and for polyclonal antibodies the use of non-immune serum of the same animal source is considered appropriate to control against non-specific binding (Dako 2006). Non-immune rabbit serum was therefore chosen as the negative control, applied at a concentration of 2µg/ml, corresponding to the known concentration of 5-LOX antibody used in this study. The concentration for antibodies against FLAP, LTA_4H and LTA_4S

were unknown. The serum was applied to sections with known positive staining, and in addition to the positive control of human tonsillar tissue. Previous work by our group has demonstrated the specificity of antibodies to FLAP, LTA_4H and LTC_4S, as described (Cowburn et al. 1998). Each immunohistochemical run included an antibody negative control for each tissue sample under interrogation, where the primary antibody was replaced by TBS.

2.2.8 Quantification of staining

Positive cells were defined as distinct nucleated cells exhibiting staining following exposure to the chromagen. Positive cells observed near the edge of a section where a wash of stain was apparent were ignored. All other immunopositive cells were counted in each tissue section and the average count in two non-adjacent sections was recorded. The total area of the counted tissue sections was measured in mm^2, ignoring sections of tissue disruption or folding, using a computer-assisted image analyser (Image Associates, Bicester UK). Immunopositive cell prevalence was documented as positive cells per mm^2 of tissue.

When epithelial staining was observed, image analysis software (Zeiss KS-400) was used (Figure 2-C) to measure percentage epithelial area staining based on red/green/colour composition as previously described (Puddicombe et al. 2000).

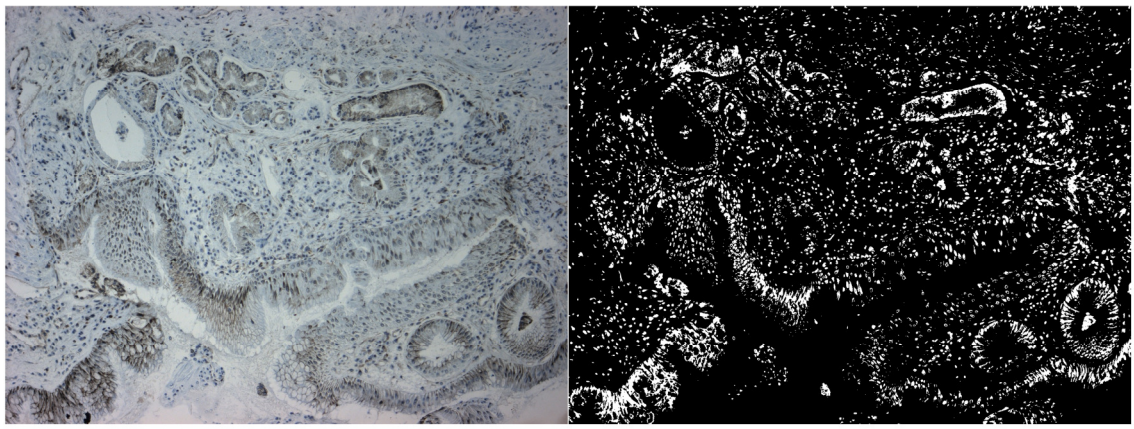


Figure 2-C: Use of image analysis software to measure percentage epithelial area staining.

2.3 Isolation, extraction, and gene expression analysis of cellular RNA using laser captured micro-dissection

2.3.1 Introduction

Laser capture micro-dissection (LCM) allows procurement of individual cellular types under direct microscopic visualisation for subsequent downstream analysis (Espina et al. 2007). Multiple steps are required with a potential for degradation of mRNA at each juncture and introduction of experimental error (Pinzani et al. 2006). The aim was to perfect this technique to isolate mRNA from laser captured samples, and to reverse transcribe and perform real time quantitative polymerase chain reaction (RT-qPCR). This technique was then used to study the association between stromal and epithelial expression of 5-LOX pathway mRNA levels between tissue types.

2.3.2 Laser capture micro-dissection

2.3.2.1 Tissue assessment

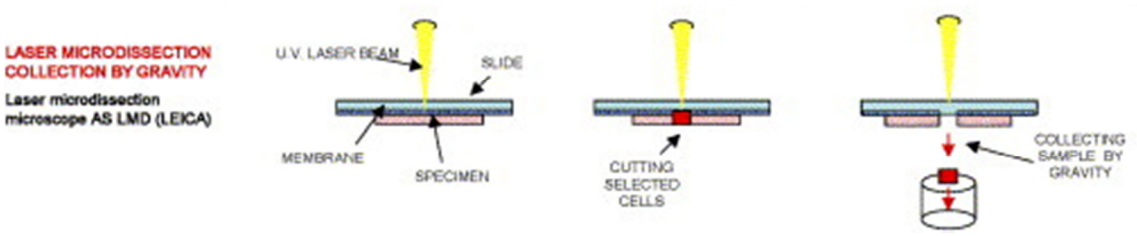
Fresh biopsy specimens were wrapped in RNase*ZAP* (Ambion, Paisley, UK) treated foil, placed in a cryo-tube, and then immediately snap frozen in liquid nitrogen. Samples were stored securely in liquid nitrogen until use. A cryo-stat (Leica CM1850UV, Leica Microsystems UK, Milton Keynes, Buckinghamshire) was pre-cooled to -30^{0}C. To ensure removal of any potential RNA contamination the cryostat along with instruments to be used for handling the samples were treated for 3 hours with ultraviolet-C rays. A sample was removed from liquid nitrogen storage, placed in the cryostat and allowed to equilibrate for 10 minutes. The sample was orientated, placed in optimal cutting medium, and placed on dry ice to allow the optimal cutting medium to harden. Sections of 8µm were cut, placed on poly-L-lysine treated slides, and allowed to air dry for 30 minutes. The section was stained with haematoxylin for 40 seconds and briefly washed in running tap water. Average cells per mm^2 were calculated for each histological subtype by use of a graticule under the light microscope. In addition, tissues were assessed to ensure the correct histological subtype, and overall tissue quality including the presence of both epithelial and stromal areas. If the biopsy fulfilled these criteria, then further sections were cut and placed on a PEN-membrane slide (Molecular Devices, Wokingham, Berkshire). A total of four sections per slide were cut, with approximately four slides per biopsy sample. Time per slide was kept to a minimum (ideally less than 1 minute), and slides were then placed immediately in a slide box (also pre-treated with RNase*ZAP*) on dry ice. The slides then either underwent a quick stain procedure (see section 2.3.2.2), or were stored in -80^{0}C in a tightly sealed box with desiccant.

2.3.2.2 Preparation of tissue sections for laser capture using quick stain technique

A quick, less intense, histological staining procedure is recommended for LCM, as warming of tissue sections even for short periods of time can allow activation of resident ribonucleases in the tissue, and result in degradation of RNA (Goldsworthy et al. 1999). The following protocol was thus developed to ensure minimum but adequate, staining of the tissue and fixing. All ethanol dilutions were made with nuclease-free water (Ambion, Life Technologies Ltd, Paisley, UK). The PEN-membrane slides were removed from dry ice and allowed to air dry for 30 seconds. They were then fixed and hydrated in 75% ethanol for 45 seconds. Cresyl violet is a hydrophilic, basic stain that binds to negatively charged nucleic acids. The resulting nuclear staining, allows for visualisation of variations in cell morphology, which is useful for identifying malignant cells. It was applied for 30 seconds, tapped off, and then the slides were then dehydrated as follows: 30 seconds in sequential order of 75%, 95%, 100%, 100% ethanol. They were then place under a fume hood and allowed to dry for approximately 1 hour before being place in a well-sealed slide box with desiccant.

2.3.2.3 Laser capture micro-dissection

A PEN-membrane slide was placed in the slide holder and mounted into the Leica Laser MicroDissector (LMD6500), which utilises gravity to allow the sample to fall into the cap of a PCR tube (certified RNase free).



Cellular areas of interest were identified, marked on screen using the computer mouse, and then dissected with the UV laser (see Figure 2-D). The area of each section cut was noted. An area was dissected to achieve a cellular capture of between 5000-10000 cells per tissue area. All samples were collected directly into lysis buffer (Ambion) or TRIzol® reagent (Invitrogen, Life Technologies Ltd, Paisley, UK), mixed by vortexing for 10 seconds, spun for 10 seconds and then placed on dry ice, prior to either immediate RNA extraction, or storage at -80^{0}C.

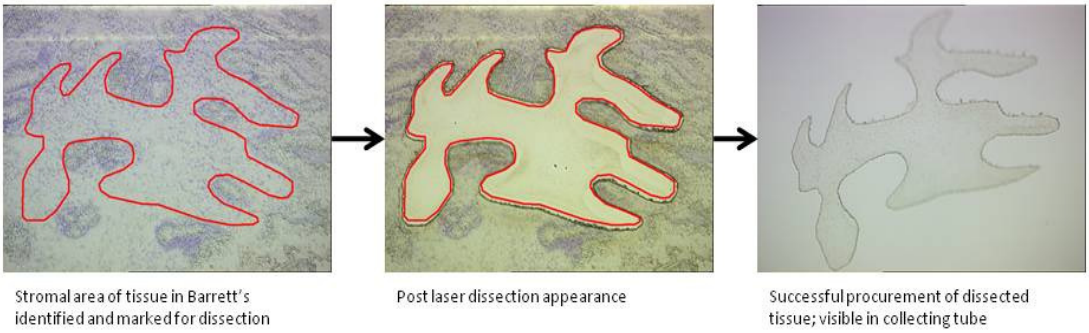
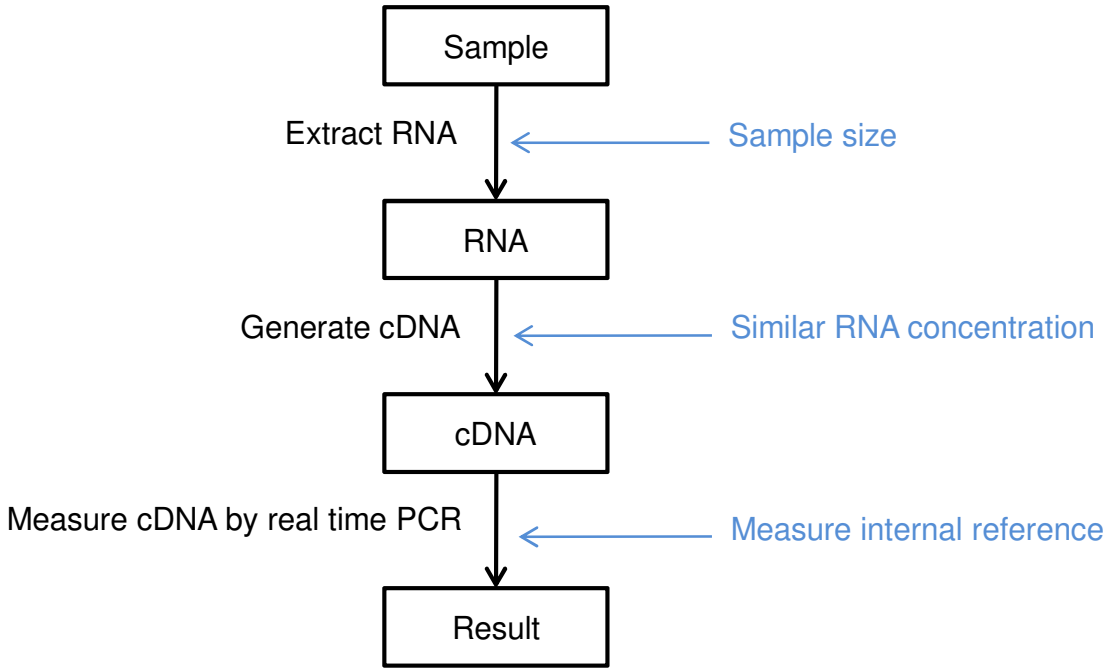


Figure 2-D: Identification, laser dissection and procurement of tissue area during laser capture micro-dissection.

2.3.3 RNA extraction and reverse transcription

2.3.3.1 Methods of normalisation

Normalisation is required at each step to control for the possibility of experimental error, which may be introduced during the multistage process required to extract and process RNA from laser captured tissue (Huggett et al. 2005; Nolan et al. 2006). The following diagram illustrates the stages at which the process was normalised in this study.



In summary, sample size was normalised by dissecting approximately 5000-10000 cells per sample. After RNA extraction, the quantity of RNA in each sample was measured and normalised to 5 nanograms/microlitre (ng/µl) prior to pre-amplification reaction (see section 2.3.3.5). Four housekeeping genes were assessed in samples (see section 2.3.4.4), and the best combination of two selected. This pair of housekeeping

genes was then included for each sample. A positive control was used as a calibrator in each run of quantitative PCR, allowing not only normalisation between samples, but also between individual runs.

2.3.3.2 Comparison of TRIzol and column based method for RNA isolation from laser captured samples

A variety of different methods for extraction of RNA are available and it is recommend that each user should optimise their own protocol to allow maximal retrieval of RNA from the LCM samples (Pinzani et al. 2006). The performances of two techniques for RNA retrieval were compared on four samples of mixed cellular material captured at LCM: extraction of RNA using TRIzol® reagent and a column based method using the RNAqueous Micro Kit (Ambion).

2.3.3.3 Isolation of RNA using a column based method

The RNAqueous-Micro kit (Ambion) uses a guanidinium thiocyanate based lysis solution, capable of disrupting cell membranes and rapidly inactivating ribonucleases. The laser dissected samples were captured into lysis solution as previous described. Captured samples stored in -80^{0}C were allowed to thaw, and then were mixed by vortexing for 15 seconds, before incubating for 30 minutes at 42^{0}C to improve cell disruption. A micro filter cartridge was assembled and 30µl of lysis buffer was applied to the centre of the filter and allowed to soak whilst the next steps were completed. After the incubation period, the samples were briefly mixed by vortexing, spun on a desk top centrifuge before 3µl of LCM additive per 100µl of lysis solution were added and mixed. Then, 0.5 volumes of 100% ethanol was added to the lysate mixture and mixed by gentle pipetting. The micro filter cartridge assembly was centrifuged at 13000 x g for 30 seconds to remove the lysis buffer from the filter. Following this the lysate/ethanol mixture was loaded onto the prepared assembly and centrifuged for 1 minute at 10000 x g to bind the RNA to the filter. 180µl of wash solution (number 1) was added to the filter and centrifuged for 1 minute at 10000 x g to pass solution through filter. A further wash solution (number 2/3) warmed to room temperature (RT) was passed through the filter by addition of a 180µl aliquot and centrifuged at 13000 x g for 30 seconds. This wash was repeated, before the filter was removed, the flow-through discarded, and centrifuged at 13000 x g for 1 minute to remove any residual fluid and dry the filter. The filter was then transferred to a fresh elution tube, and 10µl of preheated elution solution to 95^{0}C was added and stored at RT for 5 minutes, and then centrifuged at 13000 x g for 1 minute. This step was repeated with a second aliquot of elution solution. 2µl of 10x DNase buffer and 1µl of DNase were added to the elutate and incubated at 37^{0}C for 30 minutes. 2µl of DNase inactivation reagent was then added, mixed, and stored at RT for 2 minutes. The reaction was centrifuged

at 13000 x g for 1.5 minutes, and the supernatant was transferred to fresh tubes, the RNA content measured by the Nano-drop method (see section 2.3.3.5), and then stored at -80^{0}C.

2.3.3.4 Isolation of RNA using the TRIzol method

Extraction of RNA using TRIzol® reagent is well validated for the retrieval of RNA from tissues and cell preparations. Each laser captured sample was made up to 0.5 millilitres (ml) for ease of handling. The sample was mixed thoroughly by vortexing for a minimum of 30 seconds to ensure adequate cellular disruption. Chloroform (0.1 ml) was added and the sample shaken vigorously by hand for 15 seconds and then stored for three minutes at RT. The sample was then centrifuged at 12000 x g for 15 minutes at 4^{0}C to separate the solution into an aqueous and organic phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was transferred, mixed with 0.25 ml of ice-cold isopropyl alcohol to precipitate the RNA and 20 nanograms of glycogen (to help pellet visualisation), gently mixed by hand and then stored overnight at -20^{0}C. Following this overnight incubation, the solution was centrifuged to allow the RNA to collect as a pellet on the side of the tube. The prior addition of glycogen improves visualisation of the pellet. The supernatant was discarded, taking great care not to disturb the pellet, which was then washed twice with 75% ethanol and allowed to air dry for 10-15 minutes, before re-suspending in DNA free master mix (Ambion); 2µl 10x DNase 1 buffer, 1µl rDNase 1, and 17µl nuclease free water per sample. The mixture was then incubated at 37^{0}C for 1 hour. 2µl of DNase inactivation reagent was added, mixed by vortexing, stored at RT for 2 minutes, vortexing once. The reaction was then centrifuged at 13000 x g for 1.5 minutes, and the supernatant was transferred to fresh tubes, the RNA content measured by the Nano-drop method (see section 2.3.3.5), and then stored at -80^{0}C.

2.3.3.5 Quantification of RNA

The quantity of RNA in a sample may be accurately quantified using the Nano-drop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, USA). This allows a further normalisation step to be introduced prior to reverse transcription.

1µl of the RNA solution was removed and placed in 9µl nuclease free water to give a 1:10 dilution. 1µl of this sample was pipetted onto the lower measurement pedestal, the sampling arm closed and a spectral measurement made. The Nano-drop calculates absorbance at wavelength 260 nanometres (nm) and 280 nm. This allows quantification of total RNA (at 260nm) measured in nanograms per microliter (ng/µl). Using a ratio of the absorbance at 260nm and 280nm allows assessment of the purity of RNA, with a ratio of 1.8 or above indicating acceptable purity. A lower ratio may

indicate contamination with protein, phenol or other contaminants strongly absorbing at or near 280nm.

2.3.3.6 Positive control for assessment of gene expression assays and as experimental calibrator

A population of mixed granulocytes was chosen due to known expression of all of the genes of interest in this cellular type. Granulocytes were isolated from whole blood of healthy subjects. The detailed protocol may be found in the appendix C. In summary, whole blood was taken by phlebotomy from a willing volunteer and added to a collection tube with the addition of acetyl dextran solution. 15 ml of dextran were then added to this mixture and allowed to sediment for 60 minutes at RT. The separated plasma layer was removed and placed in a fresh tube, and the red cells discarded. Plasma was spun at 1150 x g for 12 minutes at RT. The supernatant was removed and a smear taken for a cell count before placing the sample in 4 ml of TRIzol reagent, and stored at -20^{0}C until RNA extraction (as previously described in section 2.3.3.4).

2.3.3.7 Generation of copy DNA for real time PCR reaction - Reverse transcription PCR

Two methods of reverse transcription were used. It was only possible to extract small quantities of RNA from laser dissected tissue, so reverse transcription with a pre-amplification step was incorporated. Higher yields of RNA were obtained from granulocytes and thus a pre-amplification step was not needed.

2.3.3.8 Reverse transcription of granulocyte RNA

2.3.3.8.1 Primer reaction Setup

Reverse transcription was performed on 1 µg of total RNA isolate using the ImPromII™ RT-PCR kit (Promega, Southampton, UK). All reverse transcription reaction set-ups contained positive (Kanamycin RNA), reverse transcription negative (RT-ve) and water controls (no-template control) to control for reagent failure, internal and external genomic DNA contamination respectively. Firstly, the RNA template and primers were mixed, as shown in table 2-A, in 0.2 ml PCR tubes and denatured at 70˚C for five minutes on a thermal cycler to remove secondary RNA structures, facilitating random/oligo dT primer annealing. At the end of the denaturing step the sample tubes were immediately placed on ice.

Reagents	Positive Control Reaction	Negative (no-template) Control Reaction	Experimental/ RT-ve Reaction
1.2kb Kanamycin Positive control RNA (1 µg)	2 µl	-	-
Experimental RNA (0.2 µg/reaction)	-	-	4 µl
Oligo(dT)$_{15}$ Primer (0.5 µg/reaction)	1 µl	-	-
Random Primer (0.5 ng/reaction)	-	1 µl	1 µl
Nuclease-Free Water	2 µl	4 µl	-
Final Volume	5 µl	5 µl	5 µl

Table 2-A: RT-qPCR Primer reaction set up table

2.3.3.8.2 RT-qPCR master mix Setup

15 µl RT-qPCR master mixes were made up as shown in table 2-B and added to the 5 µl Primer/template mixes, mixed by flicking the tubes and spun down in a bench-top mini-centrifuge.

Reagents	Positive Control Reaction (µl)	No RT Control Reaction (RT-ve) (µl)	Experimental/No-template Control Reaction (µl)
Nuclease-Free water (to final volume of 15 µl)	4.2	8.8	7.3
5 x ImPromII™ Reaction buffer	4.0	4.0	4.0
$MgCl_2$ (Final conc. 1.5 mM)	4.8	1.2	1.2
dNTP mix	1.0	1.0	1.0
RNasin Ribonuclease Inhibitor (20 U)	0.0	0.0	0.5
ImPromII™ reverse transcriptase	1.0	0.0	1.0
Final Volume	15.0	15.0	15.0

Table 2-B: RT-qPCR master mix set up table

The tubes were transferred to a thermal cycler and heated to 25˚C for 5 minutes, 42˚C for 1 hr, 70˚C for 15 minutes and then finally cooled and held at 20˚C. The copy DNA (cDNA) samples were then stored at -20˚C ready for use as templates in RT-qPCR reactions.

2.3.3.9 Pre-amplification of RNA isolated by laser capture

The Roche Lightcycler® RNA pre-amplification kit (Roche Diagnostics Ltd, Burgess Hill, UK) allows amplification of RNA, starting with 5 to 50 ng total RNA, to produce microgram quantities of cDNA that can then be used in a quantitative PCR reaction. It utilises Ribo-SPIA® technology which facilitates linear, isothermal amplification of total RNA. Linear amplification maintains the relative representation of each transcript species across samples after amplification. This is achieved by replicating only the original cDNA from RNA transcripts (not copies of cDNA copies). In addition the rate of replication is consistent for each transcript from sample to sample, thus the differential expression of transcripts after amplification reflects the differential expression of transcripts in the original starting materials.

2.3.3.9.1 Generation of first strand cDNA

First strand cDNA was first prepared from total RNA using the first strand primer mix (DNA/RNA chimeric primer mix) and transcriptor reverse transcriptase. The primers have a DNA portion that hybridizes either to the 5´- portion of the poly (A) sequence or randomly across the transcript. Reverse transcriptase extends the 3´- DNA end of each primer generating first strand cDNA. The resulting cDNA/RNA hybrid molecule contains a unique RNA sequence at the 5´- end of the cDNA strand.

A total of 5µl of RNA isolate was used, normalised to 5ng/µl. The first strand transcript enzyme mix was briefly spun on a desktop centrifuge and placed on ice. First strand buffer mix and first strand primer mix were thawed at RT, vortexed for two seconds, spun and placed on ice. Two µl of the first strand primer mix was added to 5µl of normalised sample, spun for two seconds and returned to ice. Tubes were then placed in a pre-warmed thermal cycler and incubated at 65^0C for five minutes, cooled to 4^0C and then placed on ice. First strand master mix was prepared by combining 2.5µl of buffer mix per sample with 0.5µl of transcript enzyme mix per sample, mixed by pipetting and spun down briefly and placed on ice. A reverse transcriptase negative control was included in each reaction, substituting at this point nuclease free water for the transcript enzyme mix. 3µl of first strand master mix was then added to each tube, mixed by pipetting three times, and spun. The mixture was then placed in a pre-cooled thermal cycler program to run at 4^0C for one minute, 25^0C for ten minutes, 48^0C for ten minutes, 95^0C for five minutes, cooled to 4^0C, spun for two seconds and placed on ice.

2.3.3.9.2 Generation of a DNA/RNA Hetero-duplex Double Strand cDNA

Fragmentation of the RNA within the cDNA/RNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5´- unique sequence from the first strand chimeric primers. The result is a double stranded cDNA with a DNA/RNA hetero-duplex at one end.

The second strand enzyme mix and the enhancement enzyme mix were spun for two seconds and placed on ice. Second strand buffer mix was thawed at RT, vortexed for two seconds, spun for two seconds, and then placed on ice. The second strand master mix was prepared by combining 9.75µl of second strand buffer mix per sample with 0.25µl of enzyme mix per sample, mixed by pipetting and spun down briefly and placed on ice. 10µl of second strand master mix was added to each first strand reaction tube, mixed by pipetting three times, spun for two seconds placed on ice. The reaction was then transferred to a pre-cooled thermal cycler to run at 4^0C one minute, 25^0C ten minutes, 50^0C thirty minutes, 70^0C five minutes, cooled to 4^0C, spun for two seconds and then placed on ice.

The second strand enhancement master mix was prepared by combining 1.85µl of second strand buffer mix per sample with 0.15µl of enhancement enzyme mix per sample, mixed by pipetting, spun and placed on ice. 2µl of this master mix was then added to each reaction tube, mixed by pipetting three times, spun for two seconds and placed on ice. The reaction was then transferred to a pre-cooled thermal cycler to run at 4^0C for one minute, 37^0C for fifteen minutes, 80^0C for twenty minutes, cooled to 4^0C, spun for two seconds and then placed on ice.

2.3.3.9.3 Ribo-SPIA® Amplification

SPIA® amplification is a linear isothermal DNA amplification process that uses a SPIA® DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5´- end of the first cDNA strand. This results in the exposure of a DNA sequence that is available for binding a second SPIA® DNA/RNA chimeric primer. DNA polymerase then initiates replication at the 3´- end of the primer, displacing the existing forward strand. The RNA portion at the 5´- end of the newly synthesized strand is again removed by RNase H, exposing part of the priming site for initiation of the next round of cDNA synthesis. The process of SPIA® DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of cDNA with sequence

complementary to the original RNA. An average amplification of 1,500-fold is observed with 5ng starting total RNA.

SPIA DNA/RNA chimeric primer and buffer mix were thawed to RT, vortexed for two seconds, spun for two seconds and placed on ice. SPIA enzyme mix containing DNA polymerase and RNase H was thawed on ice, mixed by inverting gently five times, spun for two seconds and then placed on ice. The SPIA master mix was prepared by combining 5µl of the buffer mix with 5µl of primer mix and 10µl of enzyme mix, per reaction. This was mixed by pipetting, spun, and placed on ice. 20ul of SPIA master mix was added to the enhanced second strand reaction, mixed by pipetting six to eight times, spun for two seconds, and placed on ice. The reaction was then transferred to a pre-cooled thermal cycler to run at 4^0C for one minute, 47^0C for one hour, 95^0C for five minutes, cooled to 4^0C, spun for two seconds, diluted 1:10, and stored at -20^0C until needed.

2.3.4 Real time quantitative PCR

2.3.4.1 Real-time PCR reaction

Eurogentec qPCR Mastermix was used for all 6-FAM™ labelled TaqMan® MGB probes, and Precision™ 2x qPCR Mastermix with SYBRgreen for PrimerDesign supplied primers. Reagents were thawed, mixed by inversion, spun down and then placed on ice. The reaction mix was prepared as follows and mixed thoroughly by inversion and spun down:

Reagent	Volume per reaction
2x Mastermix reaction buffer (+/- SYBRgreen)	10µl
20x gene expression assay or Primer	1.0µl
dH_2O	4.0µl
Total	15µl

15µl of reaction mix followed by 5µl of sample template cDNA was pipetted to each well. Each reaction was replicated in triplicate. The plate was sealed, mixed and spun down. The real time thermal cycler was programmed as follows:

Step	Cycle	Temperature	Time
Taq activation	1	95^0C	10 minutes
Quantification	40	95^0C 60^0C	15 seconds 1 minute
Cooling	1	50^0C	30 seconds

2.3.4.2 Gene expression assays

Applied Biosystem (Warrington, UK) supplied pre-designed gene expression assays to target genes of interest during RT-qPCR, with the exception of *PTPRC*. Each gene expression assay contains two unlabelled primers and one 6-FAM™ dye-labelled, TaqMan® MGB probe. The gene expression assays used are summarised in table 2-C, with the following criteria used to select the appropriate pre-designed assay:

a) Primers targeting the region encompassing the final two exons were preferred. This was to avoid polymorphisms and splice variants which tend to occur towards the beginning of the coding sequence.
b) Primers crossing boundaries were preferred due to their specificity for mRNA.
c) GenBank, a depository of nucleic acid sequences, was cross referenced for all the primers, with a higher number of GenBank mRNA transcripts preferred. This helps to avoid splice variant gene expression.
d) Length of primer can affect the efficiency of the PCR reaction, and therefore shorter primer lengths were preferred.
e) A literature search on OVID Medline was undertaken to search for previous work on the target genes under investigation, and to aid targeting of the most reliable area of the coding region of interest.

Applied Biosystem (Warrington, UK) supplied pre-designed gene expression assays to target genes of interest during RT-qPCR, with the exception of *PTPRC*. Each gene expression assay contains two unlabelled primers and one 6-FAM™ dye-labelled, TaqMan® MGB probe.
The primer to target Protein tyrosine phosphate receptor type C (*PTPRC*; RefSeq interrogated sequence - NM_002838) was obtained from PrimerDesign (Southampton, UK). The assay length was 111 and had the following sense primer: GAACAAGCATCACAAGAGTACAC,
and antisense primer: TCTTCTGTTTCCGCACTTTCTAA.

Table 2-C: Summary of gene expression assays used in real time RT-qPCR experiments

Gene Name	Gene Symbol	Chromosomal (Chr.) location of interrogated gene	Interrogated sequence (RefSeq)	Number of GenBank mRNA	Spans exon?	Amplicon size
Arachidonate 5-lipoxygenase	*ALOX5*	Chr. 10 45240414-45244218	NM_000698.2	8	Y	61
Arachidonate 5-lipoxygenase activating protein	*ALOX5AP*	Chr. 13 30228081-30236556	NM_001629.2	1	Y	80
Leukotriene A4 hydrolase	*LTA4H*	Chr. 12 94924223-94927057	NM_000895.1	7	Y	109
Leukotriene C4 synthase	*LTC4S*	Chr. 5 179153592-179155290	NM_145867.1	4	Y	80
Leukotriene B4 receptor 1	*LTB4R*	Chr. 14 23852357-23855992	NM_181657.1	1	Y	76
Cysteinyl leukotriene receptor 1	*CYSLTR1*	Chr. X 77414786-77425693	NM_006639.2	4	Y	128
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Chr. 12 6643657-6647536	NM_002046.3	121	Y	93
Beta Actin	*ACTB*	Chr. 7 5566779 -5570232	NM_001101.3	128	Y	139
Hypoxanthine phosphoribosyl-transferase 1	*HPRT1*	Chr. X 133594175 - 133634698	NM_000194.2	7	Y	100
Eukaryotic 18S ribosomal RNA	*18S*	NA	X03205.1	NA	NA	187

2.3.4.3 Validation of gene expression assays

A number of problems may occur during the PCR reaction which can affect the reliability and efficiency of each experiment. This includes formation of primer/dimers, probe degradation, template contamination, and non-specific amplification due to mis-priming or non-specific probe binding. To assess this, efficiency was calculated and melt curve experiments performed on each gene expression assay.

2.3.4.3.1 PCR efficiency

The slope of the standard curve represents the efficiency of the PCR amplification reaction, and is numerically represented by the formula: efficiency = $10^{-1/slope}$. An efficiency of 2 represents the perfect PCR reaction, which corresponds with a doubling of target nucleic acid for each cycle. The error is also calculated, which is a measure of the accuracy of the standard curve for quantification, with an acceptable value of below 0.2. The efficiency and error were calculated for each gene expression assay. A titration series of granulocytes was made from 25ng/µl to $6.10e^{-03}$ ng/µl, diluting four-fold each time. This dilution series was then combined with master mix and each of the gene expression assays to give a titration series suitable for standard curve formation.

2.3.4.3.2 Melt curve analysis

Analysis of the temperature at which the cDNA binds and separates allows us to assess the performance of the gene expression assays. In melt curve analysis, the amplified PCR product is gradually heated through a range of temperatures (e.g., from 65°C to 95°C) and SYBR green fluorescence is continuously measured. Only a single peak, which represents the specific PCR product, should be observed. The presence of other peaks indicates the presence of primer-dimers and/or nonspecific PCR products. A melt curve experiment was undertaken for each gene expression assay.
1µl of SYBR green dye was added to the PCR reaction mix of each of the titration series of granulocytes (described above), mixed by vortexing, and then the following experiment performed on the thermal cycler.

Step	Cycle	Temperature	Time
Denaturation	1	95^0C	5 seconds
Annealing	1	60^0C	5 seconds
Melt curve analysis	1	65^0C-95^0C	4.8^0C/sec
Cooling	1	50^0C	15 seconds

2.3.4.4 Housekeeping genes

Selection of a valid normalisation or endogenous control to correct for differences in RNA sampling is critical to avoid misinterpretation of results. The ideal endogenous control should have a constant RNA transcription level under different experimental conditions and be sufficiently abundant across different tissues and cell types. It is recognised that the use of one housekeeping gene is insufficient (Vandesompele et al. 2002). The following pairs of housekeeping genes were assessed: Eukaryotic 18S ribosomal RNA (*18s*), Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and Beta Actin (*ACTB*).

18s is a highly abundant ribosomal RNA and has previously been demonstrated to have good consistency across tissue types (Lossos et al. 2003). *GAPDH* and *ACTB* are commonly used and have previously been used in experiments using squamous, Barrett's and oesophageal adenocarcinoma tissue (Konturek et al. 2004; Vallbohmer et al. 2006; von Rahden et al. 2008). In a study assessing housekeeping gene variability in a variety of cancerous and normal tissues, *GAPDH*, *ACTB*, and *18s*, showed very little variability, in particular, between oesophageal squamous and carcinoma tissue (Rubie et al. 2005). De Kok et al. assessed 11 different housekeeping genes across a variety of tumour types. The authors found *HPRT1* to be the least variable single gene, and recommended it for future cancer research (de Kok et al. 2005).

Quantitative PCR was performed with the selected housekeeping genes, as previously described, on cDNA from a representation of tissue types: Squamous and Barrett's stroma, Barrett's epithelium, OA (mixed cellular type), and granulocytes. All were performed in triplicate. The difference in average crossing point value of each sample from each housekeeping gene expression assay of interest was calculated. The difference between the maximum and minimum ratio was then calculated to give the potential -fold difference in values (doubling of product for every cycle). A high value is interpreted as a large variation between pairs of housekeepers in the different tissue types, and thus unreliable. The pair with the lowest value was chosen for use in the final analysis.

2.3.4.5 Analysis of the purity of laser captured epithelial cell populations

PTPRC gene is known to be expressed exclusively in cells of haematopoietic origin with the exception of erythrocytes. The expression of the *PTPRC* gene was assessed in laser captured samples of epithelial area of tissue, to assess the purity of these cell populations.

2.3.4.6 Controls

A reverse transcription negative control and a no template (nuclease free water) were included in each run.

2.3.4.7 Relative quantification of samples using the E-method

Relative quantification of gene expression was used to quantify differences in the expression level of the target genes between the different samples of tissue. The quantity of the target gene and the reference (housekeeping) genes within the same sample were determined, and results expressed as a ratio. By calculating the ratio in this way one can correct for differences in quality and quantity between samples, caused by, for example, variations in cDNA synthesis efficiency. A calibrator (granulocytes) was included in each separate real time qPCR reaction enabling normalisation for run to run differences. The final result was expressed as a normalised ratio and is summarised by the following equation:

$$\text{Normalised ratio} = \left[\frac{\text{conc. of target}}{\text{conc. of ref sample}}\right] : \left[\frac{\text{conc. of target}}{\text{conc. of ref calibrator}}\right]$$

In addition, rather than assuming an efficiency of two for each target gene (which can produce incorrect gene expression data), standard curves and efficiencies generated during the validation experiments were used.

2.4 Statistical analysis and choice of statistical test

Statistical tests were performed with PASW Statistics version 18 for Windows (IBM UK Ltd, Middlesex, UK). For each dataset a histogram was plotted to assess distribution, and skewness and standard error of skewness calculated. Skewness is a measure of the asymmetry of a distribution. If the actual value of skewness falls outside plus or minus twice the standard error of skewness, then the distribution is said to be significantly skewed. If datasets were non-parametric then values were $\log_{10}$-transformed and tests of Normality repeated.

Unpaired Student's t-tests were then used for comparison between groups on original or $\log_{10}$-transformed values as appropriate, dependent on the data distribution determined by Normality testing. Equal variance was not assumed.

Counts of cells immunopositive for each 5-LOX pathway protein were also correlated with counts of neutrophils, eosinophils, monocyte-macrophages and T-lymphocytes) using Pearson's correlation coefficient.

For laser captured samples, where only small numbers of samples were present in each group, the Mann-Whitney-U was utilised.

Dr CPC Boger

CHAPTER 3: Cost-utility analysis

In this chapter the methodology for the cost-ultility analysis is presented. The aim of this study was to assess whether radiofrequency ablation (RFA) of Barrett's metaplasia (BM) containing high grade dysplasia (HGD) is a cost-effective alternative in the UK compared with oesophagectomy. Section 3.1 reviews existing cost-effective analyses on ablative therapies for BM. The research question is defined in detail in section 3.2 using the PICO framework and section 3.3 documents the construction of a decision analytic model. A purposive review of the literature to identify parameters for use in the cost-utility analysis is found in sections 3.4 (transition probabilities) and section 3.5 (utilities). The calculated costs are presented in section 3.6 and methods for deterministic and probabilistic analysis in section 3.7.

3.1 Review of Existing cost-effective analyses on ablative therapies for Barrett's metaplasia

Prior to this thesis two techniques for ablating BM had been the subject of cost-effectiveness studies; RFA and photo-dynamic therapy (PDT).

3.1.1 Radiofrequency Ablation

Das et al. (2007) used a Markov model to compare the cost-effectiveness of the following strategies in a 50 year old with non-dysplastic BM: (i) no intervention (natural history), (ii) surveillance alone, (iii) RFA. The model was run until death of the entire cohort. It is not clear how parameters, costs and utilities were chosen. They used an estimate of 50% for complete removal of intestinal metaplasia (CR-IM) for RFA, lower than the published studies have reported. The discount rate was not stated. RFA resulted in an additional 0.42 quality adjusted life years (QALYs) at a cost of $46882 compared with no intervention, and 0.14 QALYs at a cost of $69270 compared with surveillance alone. They concluded that patient age, cost of RFA, and CR-IM were critical determinants of the cost-effectiveness of RFA.

Inadomi et al. (Inadomi et al. 2007) used a Markov model to compare the cost-effectiveness of the following strategies in a 50 year old with low-grade dysplasia (LGD) in BM: (i) no intervention, (ii) surveillance, (iii) oesophagectomy, and (iv) RFA. It is not clear how parameters, costs, and utilities were chosen. The discount rate was not stated. RFA was the most cost-effective strategy resulting in an additional 1.8 QALYs at an incremental cost of $9842 compared with no intervention, 0.89 QALYs at an

incremental cost of $2376 compared with surveillance. Oesophagectomy was eliminated through dominance. The incremental cost-effectiveness ratio (ICER) was $5468/QALY versus no intervention, and $2669/QALY versus surveillance.

3.1.2 Photodynamic therapy

Three cost-effective analyses were identified assessing other management options for HGD in BM. They were all undertaken prior to the introduction of RFA as a treatment option.

Hur et al. (2003) constructed a Markov model to compare the cost-effectiveness of the following strategies in a 55 year old male with HGD in BM: (i) PDT with surgical oesophagectomy, (ii) intensive surveillance strategies. The model was run until death of the entire cohort. There was no literature review supporting selection of the parameters. Utilities were derived from patients using visual analogue scales (VAS), which are associated with measurement biases (Drummond et al. 2005). Costs were derived from United States (US) estimates. They used a discount rate of 30% (the NICE reference case recommends 3.5%). PDT was the most cost-effective strategy resulting in an additional 1.65 QALYs at a cost of $20,400 compared to surveillance, and 2.17 QALYs at a cost of $7,100 compared to oesophagectomy. The ICER was $12,400/QALY versus surveillance, and $3,300/QALY versus oesophagectomy.

Vij et al. (2004) constructed a Markov model to compare the cost-effectiveness of the following strategies in a 55 year old male with HGD in BM: (i) surveillance, (ii) oesophagectomy, (iii) PDT followed by oesophagectomy for residual HGD, (iv) PDT followed by surveillance for residual HGD. The model was run until death of the entire cohort. Parameters were obtained from a structured review of the literature. The majority of utility values were estimated by the authors. Costs were from US estimates from a 3rd party payer perspective. They used a discount rate of 3%. PDT followed by surveillance was the most cost-effective strategy resulting in an additional 0.49 QALYs at an incremental cost of $23255. The ICER was $47410/QALY, with surveillance and PDT followed by oesophagectomy for residual HGD eliminated through extended dominance.

Shaheen et al. (2004) used a linear decision tree terminating in a Markov model (for those not undergoing oesophagectomy) to compare the cost-effectiveness of the following strategies in a 50 year old male with HGD in BM: (i) no surveillance, (ii) surveillance, (iii) oesophagectomy, (iv) PDT. The surveillance strategy was halted at 80 years of age or death, but it is unclear what the time horizon was after this for the other strategies. It is unclear how the parameters were chosen. Utilities were derived

from patients using VAS. Costs were from US estimates from a 3rd party payer perspective. They used a discount rate of 3%. PDT was the most effective strategy resulting in an additional 1.61 QALYs at an incremental cost of $41250 compared with no surveillance. The ICER was $25621, with oesophagectomy dominated by no preventative strategy, and surveillance eliminated through extended dominance.

3.1.3 Summary

Although some parallels between the Markov models used in the PDT cost-effectiveness studies can be drawn to ours, the detail on the precise health states used is difficult to ascertain from the studies, and thus it was appropriate that a new model was constructed. Only Vij et al. (2004) conducted a structured review of the literature to ascertain transition probabilities, with the majority of health states estimated by the authors. Further data may be available and thus a literature search was conducted.

3.2 Defining the research question

The PICO framework (Richardson et al. 1995) was used to define the research question in more detail.

3.2.1 Population

The model consisted of a hypothetical cohort of 100 male patients aged 64 years, the mean age of patients undergoing oesophagectomy for HGD in published trials over the last 10 years (Nigro et al. 1999; Patti et al. 1999; Nguyen et al. 2000; Zaninotto et al. 2000; Headrick et al. 2002; Tseng et al. 2003; Reed et al. 2005; Sujendran et al. 2005; Chang et al. 2006; Prasad et al. 2007; Williams et al. 2007). BM is predominantly a disease of men with a male to female ratio of approximately 9:1 and therefore it is appropriate that the cohort contained male patients only (Blot et al. 1991). The following pre-treatment assessment was assumed for both strategies: endoscopy had been undertaken on two occasions revealing BM with HGD, and any visible nodules, masses or surface irregularities had been removed by endoscopic mucosal resection (EMR). Patients found to have OA after EMR were excluded from the analysis. It was assumed that patients were fit for both treatment modalities. The model included patients with either uni- or multifocal HGD.

3.2.2 Intervention

A number of management strategies for treating HGD in BM exist in the UK. In the UK, surveillance of those with HGD is not presently undertaken. The review of previous cost-effective analyses suggested that PDT was an expensive although effective treatment option for BM (see section 3.1.2). However, in the UK RFA is rapidly

superseding PDT, as PDT is associated with treatment-related adverse events in up to 94%, a stricture rate as high as 37%, and ultimate progression to cancer in 15% (Overholt et al. 2005; Overholt et al. 2007). Although PDT is still practiced, and newer photosensitising agents are under investigation, RFA has clinically become the method of choice for ablating Barrett's oesophagus; thus PDT is not included in the model (see section 1.8).

3.2.3 Comparator

Current UK guidance recommends that for patients with persistent HGD despite intensive acid suppression, oesophagectomy be performed in a specialist centre in those fit for surgery (Watson et al. 2005). Oesophagectomy is therefore the comparator as it constitutes the "gold standard" treatment in the UK. It was assumed that patients are fit for both treatment modalities, although it is acknowledged that some will be fit for RFA, but not oesophagectomy.

3.2.4 Outcomes

In line with the NICE reference case a cost utility analysis was used to analyse the cost-effectiveness of radiofrequency ablation. A *de novo* model was constructed, and the following outcomes ascertained:

Primary

- Cost/incremental cost
- Effectiveness measured in QALY/incremental effectiveness
- IECR
- Cancer incidence
- Complication incidence
- Mortality and survival

Secondary

- Deterministic sensitivity analysis - to identify key data points associated with uncertainty
- Probabilistic sensitivity analysis - to explore the uncertainty surrounding the input parameters.

3.3 Construction of a decision analytic model

3.3.1 Model overview

The model was constructed to include major procedural and post-procedural complications and mortality as suggested by review of the literature. At the end of each cycle, which corresponded to 6 months, patients progress to a health state depending on the derived transition probabilities. A utility and cost was attributed to

each state, which patients would accumulate as they progressed through the model. The analysis followed the cohort for 25 years or to death. Costs were estimated from an NHS perspective and outcomes from the viewpoint of the patient. All costs and benefits were discounted at 3.5% as recommended by NICE (2008).

3.3.2 Explanation of model and health states

3.3.2.1 Treatment strategy - Oesophagectomy

In this strategy, patients with confirmed HGD in BM underwent immediate oesophagectomy. A transition state diagram for the oesophagectomy strategy is illustrated in figure 3-A.

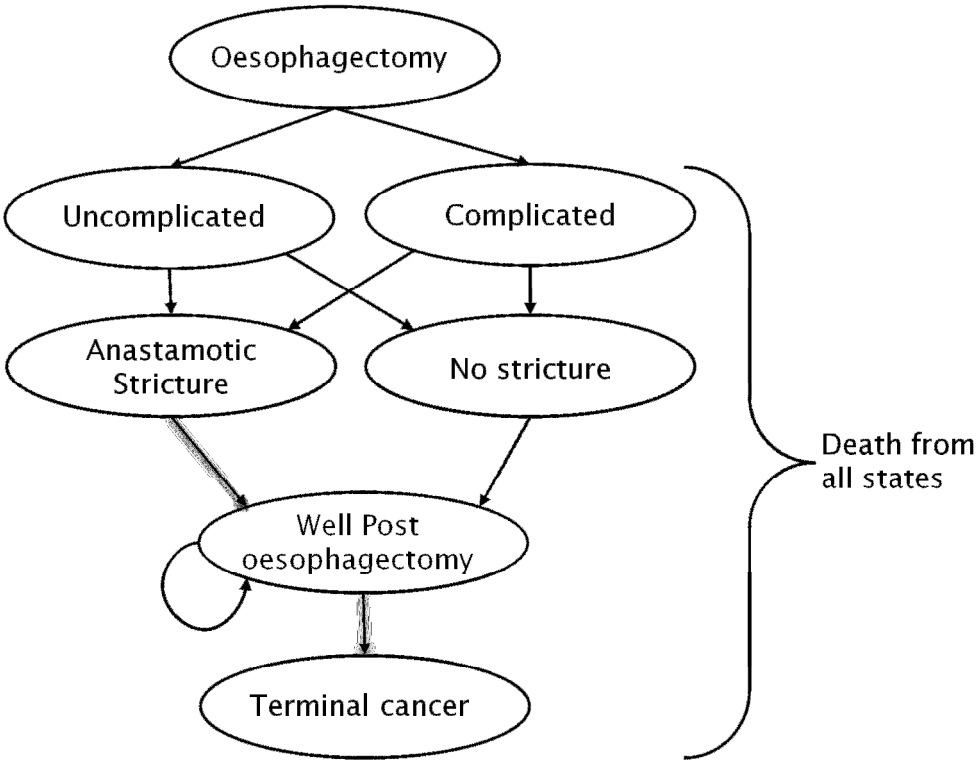


Figure 3-A: Transition state diagram (Markov model) of oesophagectomy strategy
The health states are shown with arrows showing how patients can progress between states at the end of each cycle.

3.3.2.1.1 Complicated/Uncomplicated oesophagectomy

Patients may suffer significant complications from oesophagectomy requiring an intensive care unit stay, increasing cost and reducing quality of life. Complications were defined as those requiring an intensive care unit stay, and included anastamotic leaks, respiratory distress, significant wound infections or dehiscence, cardiac arrhythmia, and delirium.

3.3.2.1.2 Anastamotic stricture/No stricture

The literature demonstrates that post-operative anastamotic strictures are a significant problem in the first year post oesophagectomy (Headrick et al. 2002). Patients remained in this health state for two cycles to represent this.

3.3.2.1.3 Well post oesophagectomy and progression to terminal cancer

There are no further treatment options available if a patient develops recurrent OA post oesophagectomy. In this scenario it was assumed that they entered a 6-month terminal cancer phase, representing the median survival time with inoperable oesophageal cancer, and allowing the modelling of costs and measures of quality of life experienced by this group of patients, such as palliation of dysphagia with oesophageal stenting. Conversely, if a patient did not progress to terminal cancer during the first 5 years post-oesophagectomy then they were considered cured.

3.3.2.2 Treatment strategy - Radiofrequency ablation

In this strategy, patients with confirmed HGD in BM underwent a treatment course of RFA with the HALO device (Bârrx Medical, Sunnyvale, California, USA), as described in section 1.8.3. Published experience to date suggests an average of one HALO^{360} and two HALO^{90} sessions are needed per patient to achieve complete removal of the Barrett's segment and replace it with a neo-squamous lining in patients with HGD (Gondrie et al. 2008a). A transition state diagram for the RFA strategy is illustrated in figure 3-B.

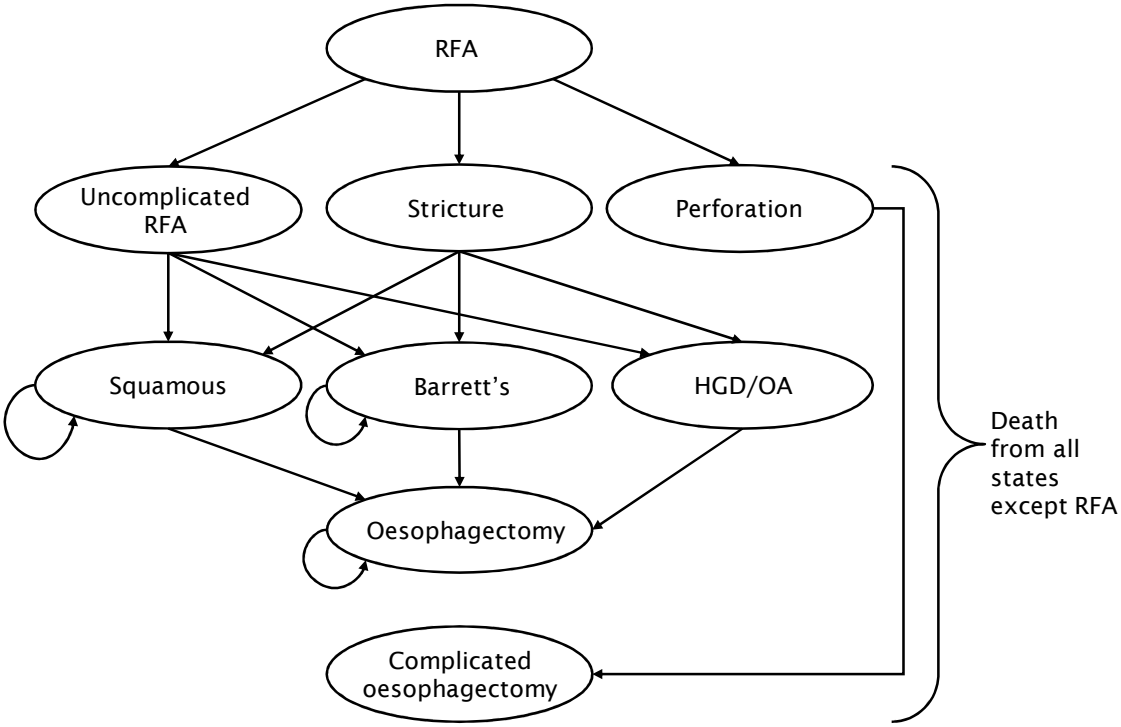


Figure 3-B: Transition state diagram (Markov model) of RFA strategy
The health states are shown with arrows showing how patients can progress between states at the end of each cycle.

3.3.2.2.1 Uncomplicated RFA, stricture or perforation

Patients undergoing RFA may experience an oesophageal perforation, and in the model it was assumed that they either died or underwent an immediate oesophagectomy. In clinical practice, perforation may be treated medically, but given that the patient has an unknown histological outcome from RFA with the possibility of persistent HGD, oesophagectomy is likely to be the treatment option of choice.

It was assumed that patients experiencing a stricture will either die or progress to one of the RFA outcome states (neo-squamous, Barrett's, persistent HGD). In practice this group may develop a perforation as a complication of oesophageal dilatation, but the risk of this is low (Quine et al. 1995). This risk is excluded from the oesophagectomy model, to ensure equality between groups.

3.3.2.2.2 Surveillance strategy for post RFA health states (squamous or BM)

Currently, there is no fixed surveillance strategy for patients with neo-squamous and BM post-RFA. For the base case analysis the following strategy was assumed: if neo-squamous oesophagus is found after RFA, then yearly endoscopy was performed for 5-years, and if free of BM or worse pathology after that time, no further endoscopic follow-up was undertaken. If the patient had BM after RFA treatment then yearly

endoscopy was performed for the first 5-years, and if free of HGD or worse at that time, endoscopic surveillance continued on a 2-yearly basis as per current UK practice. In the scenario analysis this screening protocol was altered to analyse what effect it would have on the outcome of the cohort.

If HGD or OA is picked up during the surveillance strategies, it was assumed the patient undergoes an oesophagectomy. This part of the RFA model has the same structure as the oesophagectomy strategy. It was assumed that the risk of developing cancer in the neo-squamous oesophagus is most probable during the first 5-years post treatment, and thus after this period (as with the post oesophagectomy group), the patient was considered cured.

3.3.2.2.3 Treatment failure – HGD/OA post RFA

There are no data on the outcome of those with treatment failure post RFA. For this parallels were drawn with data from other ablative therapies (PDT) with longer term follow up available. This demonstrates that treatment failures were still operable at time of progression, and revealed disease not extending beyond the submucosa (T1) on post-operative staging (Prasad et al. 2007). On this basis, in the model those with post RFA treatment failure had operable disease but with a reduction in 5-year survival consistent with the predicted more advanced disease.

3.4 Transition probabilities

3.4.1 Overview

Parameter values for transition probabilities and utilities were obtained from peer reviewed literature. The Medline database from January 1999 to May 2009 was searched, as well as abstracts from Digestive Diseases Week and the British Society of Gastroenterology Annual Meeting. Bibliographic lists of relevant articles were searched, and in addition a list of relevant papers from the manufacturers of the HALO device was obtained. The most suitable study(s) were chosen to provide the parameters for the base case, by using data from randomised controlled trials if available. If not, available data were chosen from well-constructed observational (cohort) studies, with inclusion criteria similar to those in the required hypothetical cohort. Assessment was also made of inclusion criteria, baseline assessment, size, duration of follow-up, loss to follow-up and assessment of outcome. In addition to the base case values a plausible range (high/low values) was selected for use in the sensitivity analysis. This range was derived from variations in the published literature, or by consensus of four researchers when information was not available or there was considerable uncertainty in the literature. All transition probabilities used in the model are shown in table 3-A.

Five key areas of where transition probabilities were required were identified:

- Oesophagectomy for HGD (section 3.4.1.1)
- Progression to terminal cancer after oesophagectomy for RFA treatment failure (section 3.4.1.2)
- Radiofrequency ablation for HGD (section 3.4.1.3)
- Progression post RFA (section 3.4.1.4)
- Mortality from oesophageal perforation or dilatation (section 3.4.1.5)

Full search strategies may be found in the appendix D.

3.4.1.1 Oesophagectomy for HGD

Parameters required:

i) Oesophagectomy complications (peri-operative and anastamotic stricture)
ii) Mortality
iii) Progression to terminal cancer post oesophagectomy

The search strategy identified 12 relevant studies. Of these six were excluded for the following reasons:

- Inclusion of patients with pre-operative diagnosis of OA (Nigro et al. 1999; Zaninotto et al. 2000; Romagnoli et al. 2003; Chang et al. 2006)
- Inappropriate cohort - assessment of specific surgical technique (Nguyen et al. 2000), those with GORD only (Patti et al. 1999).

i) Oesophagectomy complications

There were difficulties in ascertaining complication rates due to lack of definition in some studies as to the criteria used for inclusion as a complication. This may explain the wide variation in rate, although other factors such as surgical technique, and differences between centres, may also play a role.

- Peri-operative complications

The study by Prasad et al. contains the largest cohort of patients over the most recent time frame (1994-2004), and has inclusion criteria similar to the cohort (i.e. HGD with pre-operative EMR as appropriate), and thus is used to form the base case. The studies by Tseng et al. (morbidity; 29%) and Headrick et al. (57%) represent the range of values found from the literature search and thus form low and high values respectively.

- Anastomotic stricture

The study by Headrick et al. contains the most complete data on post oesophagectomy stricture formation (stricture rate; 51%) and is thus used to form the base case. The studies by Prasad et al. (12.6%) and Williams et al. (82%) represent the range of values found from the literature search and thus form low and high values respectively.

ii) Mortality

The values found from the literature search for mortality from oesophagectomy for HGD were lower than expected with a range of 0-2%. To allow for this uncertainty a study by Portale et al. (2006) was included which examined mortality after oesophagectomy for oesophageal cancer, and found a mortality of 4.5%. This concurs with recently published national UK audit figures (available after the model was run), which demonstrate a 30-day mortality rate of 3.1% with a range of 2.0-4.5% (Palser et al. 2009). The study by Prasad et al. (2007) was chosen to form the base case for previously documented reasons.

iii) Progression to terminal cancer post oesophagectomy

Only studies with adequate follow up were chosen and where actual numbers of patients were stated. The model takes into account age related mortality and thus data was extracted from the relevant studies so that 6-monthly transition probabilities could be calculated. Two studies had a follow up period of over 5-years (Headrick et al. 2002; Prasad et al. 2007). In the study by Headrick et al., four died from metastatic oesophageal cancer, and a further three from metastatic cancer from other primary sites. In the study by Prasad et al., two in the oesophagectomy group died from cancer (metastatic transitional cell and malignant astrocytoma) but none of oesophageal cancer, which is in contrast to the study by Headrick et al. and also the other two studies with reasonable follow up data (Tseng et al. 2003; Reed et al. 2005). The base case parameter was estimated to take account of this discrepancy in the literature. The studies by Prasad et al. (5-year survival; 100%) and Headrick et al. (92.9%) represent the range of values found from the literature search and thus form low and high values respectively.

3.4.1.2 Progression to terminal cancer after oesophagectomy for RFA treatment failure

As stated previously there are no data on the outcome of those with treatment failure post RFA, and this study assumed that patients undergoing oesophagectomy for RFA recurrence will have T1 disease. It was therefore necessary to conduct an additional literature search to identify survival data for those undergoing oesophagectomy for OA.

The search strategy identified 12 relevant studies. Of these nine were excluded for the following reasons;

- No breakdown of survival by stage of tumour (Hofstetter et al. 2002)
- Inclusion of patients with squamous cell carcinoma (Mariette et al. 2004; Altorki et al. 2008; Pennathur et al. 2009)

- Follow up period insufficient (Johansson and Walther 2000)
- Insufficient data to calculate disease free survival (Stein et al. 2000; Hagen et al. 2001; Hofstetter et al. 2002)
- Inappropriate cohort - assessment of specific surgical technique (Yamamoto et al. 2005), inclusion of patients whom they waited until had symptoms (Romagnoli et al. 2003)

The study by Prasad et al. contains data from a cohort of patients who have undergone oesophagectomy after progression of their disease after PDT. It is thus a similar scenario to the model in this study and is the most appropriate for the base case parameter. The study by Portale et al. provided data from a large cohort who underwent oesophagectomy for adenocarcinoma of varying stages. The breakdown of data for those with T1 disease is used to form the high value. For the low value a 5-year survival of 100% was assumed.

3.4.1.3 RFA for HGD

Parameters required:

i) Outcomes after RFA (squamous-lined oesophagus, Barrett's oesophagus without HGD, HGD or OA).

ii) RFA complications (no complications, perforation, stricture requiring dilatation).

The search strategy identified eight relevant studies. Of these four were excluded for the following reasons;

- Data included in more recent publication (Gondrie et al. 2008a; Gondrie et al. 2008b)
- Observational data with no fixed end point and insufficient follow up (Ganz et al. 2008)
- Insufficient number with HGD (Roorda et al. 2007; Hernandez et al. 2008)

i) Outcomes after RFA

The randomised control trial (RCT) by Shaheen et al. (Shaheen et al. 2009) is the highest quality study and thus data was extracted from this study to form the base case estimates of outcome after RFA treatment. The studies by both Pouw et al. (2008) and Sharma et al. (2009) are limited by their methodological design (observational, variation in inclusion criteria, different treatment modalities), but data representative of the cohort were extracted, and included to represent the uncertainty of outcome for Barrett's health states post RFA.

ii) RFA complications

- Stricture after RFA

The RCT by Shaheen et al. (2009) is again chosen for its strength of methodological design. The study by Pouw et al. (2008) included a number of patients who had undergone extensive EMR which may explain the higher stricture rate. However, the model presented here allowed for prior EMR, and thus this higher figure of 9% is used to form the high value, with the low value (0%) derived from the study by Sharma et al. (2009).

- Perforation after RFA

To date no oesophageal perforations have been documented in published trials. The manufactures of the HALO system were contacted directly for a summary of cumulative worldwide safety data. As of April 2009, eight perforations had occurred out of approximately 16000 patients treated with RFA worldwide. This parameter was therefore used to form the base case estimate and the high value, with the 0% perforation rate quoted in the published literature forming the low value.

3.4.1.4 Progression post RFA

Parameters required

i) Progression post RFA from neosquamous oesophagus to HGD/OA

ii) Progression post RFA from BM to HGD/OA

A review of the studies identifying patients with HGD in BM undergoing RFA, demonstrated that there are no data available describing annual risk of HGD in patients after successful RFA therapy. Assumptions have thus been made based around known progression rates of those with treatment-naive BM.

A systematic review was undertaken in 2006 as part of the health technology appraisal (HTA) report on surveillance in BM (Garside et al. 2006). Subsequently, an important additional study was published which reported data on the natural history of BM in the largest cohort of patients (n=618) published to date (Sharma et al. 2006). During the mean follow up of 4.12 years, 22 patients developed HGD resulting in an incidence of 0.9%/year (95% confidence intervals, 0-1.7%).

i) Progression post-RFA from neosquamous oesophagus to HGD/OA

The precise annual risk of development of HGD or cancer in a patient with neo squamous oesophagus post RFA or BM after eradication of HGD by RFA is not known. Results so far have suggested that up to 6.8% of patients may have areas of non-visible islands of intestinal metaplasia, termed sub-squamous IM, detected on random biopsy, despite an apparent lack of IM on endoscopic visualisation (Shaheen et al. 2008b). The

concern is that these "hidden" areas of IM have a risk of progression to HGD/cancer at a later date. Adjustments to account for this were made.

The risk of developing HGD or OA in patients with neo-squamous oesophagus was estimated using published data of having residual subsquamous intestinal metaplasia (Shaheen et al. 2009). The low value assumed no risk of residual subsquamous intestinal metaplasia and the high value assumed a higher risk of having non-visible islands of BM found with previous ablative therapies, and assuming the residual BM contained HGD (Overholt et al. 2003).

ii) Progression post RFA from BM to HGD/OA

Here it is assumed that patients with BM post-RFA have the same probability of progression to HGD as patients with treatment naive BM.

3.4.1.5 Mortality rate after oesophageal perforation or dilatation for oesophageal stricture

Parameters required:

i) Death from iatrogenic oesophageal perforation
ii) Death from oesophageal dilatation

The search strategy identified four relevant studies. Of these three were excluded for the following reasons:

- Incomplete follow up data (Hernandez et al. 2000)
- Inclusion of non-endoscopic perforations (Fernandez et al. 1999)
- Unclear differentiation between benign and malignant disease (Adamek et al. 1997)

The study by Quine et al. (1995) is a UK multicentre prospective audit, and is the only study to differentiate clearly between benign and malignant disease, and it is therefore used to form the base case and the low/high values.

In the model, patients who perforate do so from a new treatment modality (RFA) with no defined outcomes, and thus a deliberately wide range of values was used to represent this uncertainty.

3.4.2 Data extraction strategy

Data were extracted, endeavouring to obtain actual numbers where possible. Annual risks and 5-year survival data were converted to 6-monthly probabilities using the following validated formulae (Briggs et al. 2006), with the assumption that 6-monthly progression rates were constant:

$$R = -[\ln(1 - P)] / t$$

where R = rate, P = probability in particular time frame and t = time frame.

Description	Base case estimate	Range	Distribution	Source - Author (Year)
RFA complications				
No complications	0.94	0.90-1	Jointly determined Dirichlet	Pouw (2008), Shaheen (2009), Sharma (2009); assumption
Perforation	0.0005	0-0.005		
Stricture requiring dilatation	0.06	0-0.09		
Outcomes after RFA				
Neo- squamous lined oesophagus	0.82	0.45-0.91	Jointly determined Dirichlet	Pouw (2008), Shaheen (2009), Sharma (2009); assumption
Barrett's oesophagus without HGD	0.11	0.09-0.25		
HGD or adenocarcinoma	0.08	0-0.30		
Progression probabilities during post RFA surveillance (per cycle – 6 months)				
Neo-squamous to HGD/OA	0.0002	0-0.0027	Beta ($\alpha = 1, \beta = 4596$)	Overholt (2003), Garside (2006), Sharma (2006)
Barrett's to HGD/OA	0.0068	0-0.0085	Beta ($\alpha = 4, \beta = 953$)	
Oesophagectomy complications				
Peri-operative complications	0.37	0.27-0.57	Beta ($\alpha = 26, \beta = 44$)	Headrick (2002), Tseng (2003), Prasad (2007)

Table 3-A: Summary of Transition probabilities

Summary of the base case estimate and range of transition probabilities used in the model.

Description	Base case estimate	Range	Distribution	Source - Author (Year)
Anastamotic stricture	0.51	0.13-0.83	Beta ($\alpha = 27$, $\beta = 26$)	Headrick (2002), Williams (2003), Prasad (2007)
Progression to adenocarcinoma post oesophagectomy				
After oesophagectomy for HGD	0.001	0-0.007	No distribution	Headrick et al. 2002; Tseng et al. 2003; Reed et al. 2005; Prasad et al. 2007
After oesophagectomy for post-RFA recurrence	0.0057	0-0.021	Beta ($\alpha = 18, \beta = 3239$)	Portale et al. 2006; Prasad et al. 2007
Mortality				
Death from iatrogenic oesophageal perforation	0.35	0-0.5	Beta ($\alpha = 2, \beta = 15$)	Quine et al. 1995
Death from oesophageal dilatation	0.005	0-0.01	Beta ($\alpha = 3, \beta = 551$)	Quine et al. 1995
Death from oesophagectomy	0.014	0-0.045	Beta ($\alpha = 1, \beta = 69$)	Tseng et al. 2003; Reed et al. 2005; Portale et al. 2006; Prasad et al. 2007; Williams et al. 2007

Table 3-A continued

3.5 Utilities

Utilities are cardinal values that represent the strength of an individual's preference for specific outcomes under conditions of uncertainty. In health economic modelling they allow a value to be placed on a particular health state, enabling the generation of QALYs, used as the outcome measure in the model.

A number of methods are available for measuring utility. The visual analogue scale (VAS) consists of a line on a page with clearly-defined end-points, with or without marks on the line. Subjects are asked to specify their level of agreement to a statement (such as description of a health state scenario) by indicating a position on this line. They are however subject to bias. These include end-of-scale biases where subjects tend to avoid using ends of the scale regardless of how good or bad a health state is, and context bias in which subjects space out the outcomes over the scale regardless of how good or bad the states are (Drummond et al. 2005). The standard gamble is the preferred method as it avoids these measurement biases. The standard gamble approach involves presenting individuals with a choice between two alternatives: a health state that is certain and a gamble with one better and one worse outcome possible. Respondents are asked what probability of the better outcome would make them indifferent between remaining in the described health state for certain or going for the risky option.

A literature search was performed to identify studies that measured quality of life in the required chosen health states and relevant values extracted. It was not possible to obtain utility values for all model states. Therefore known recorded values were used to provide a framework in which unknown values were estimated. Low and high values were derived either from 95% confidence intervals if available, or estimated to allow for the uncertainty surrounding the individual parameter. Derived utilities are shown in table 3-B.

Description	Base case estimate	Range	Distribution	Source Author (year)
RFA without complications	0.94	0.84-1	Beta (α = 31, β=2)	Assumptions based on de Boer (2002) and Hur (2006)
RFA complicated by stricture	0.92	0.82-1	Beta (α = 31, β=3)	
Post RFA with neo-squamous oesophagus	0.97	0.9-1	Beta (α = 42, β=1.3)	
Post RFA with Barrett's oesophagus	0.95	0.883-1	Beta (α = 50, β=3)	
Oesophagectomy without complications	0.92	0.77-1	Beta (α = 19, β=2)	de Boer (2002)
Oesophagectomy with complications	0.91	0.66-1	Beta (α = 9, β=1)	
Anastamotic stricture post oesophagectomy	0.92	0.841-0.999	Beta (α = 41, β=4)	Hur (2006)
Fully recovered post oesophagectomy	0.96	0.89-1	Beta (α = 46, β=2)	de Boer (2002)
Terminal oesophageal cancer	0.34	0.03-0.65	Beta (α = 3, β=5)	

Table 3-B: Summary of health state utilities used in the model

The base case estimates, range of utilities, and the distributions used in the probabilistic sensitivity analysis used in the model are shown..

3.5.1 Utility of oesophagectomy strategy disease states

Three relevant studies looking at quality of life issues in patients with HGD who had undergone oesophagectomy were identified (Headrick et al. 2002; Chang et al. 2006; Moraca and Low 2006). All used a multi-attribute health status classification system (the SF-36) not utility values, and thus were not transferable to the required health states .

Only one study that used utilities to measure patient preference was identified (de Boer et al. 2002). Although it focused on oesophagectomy for oesophageal cancer, parallels could be drawn to the health states defined in this study. Due to the importance of this study in the derivation of the utility values in this study, it is summarised below.

This study set in Holland collected patient utilities for health states after transhiatal or transthoracic oesophagectomy for oesophageal cancer. Information was collected by two researchers 3-12 months after surgery. They excluded those with inoperable disease at the time of operation and those with recurrence, and thus the final cohort consisted of patients cured of cancer by oesophagectomy. Patients were trained in standard gamble methodology, and then at interview, asked to rate their own health state and additionally were given one of seven health state scenarios. They excluded those with insufficient cognitive ability to complete the study. Both VAS and standard gamble were used.

Out of the 93 initially consented, 13 died, 21 had recurrent disease, 6 refused participation and 3 could not be reached. A total therefore of 50 patients were interviewed. The results are summarised in table 3-C.

One can approximate health state scenarios from the study by de Boer et al., to the health states in this study, as follows:

- Home, disease free *approximates to* well post oesophagectomy.
- Irresectable *approximates to* terminal oesophageal cancer.

Additionally, to calculate the utility for post oesophagectomy health states in this study (to make allowance for hospital stay and the recovery period at home) utility values were used from the study by de Boer et al. from the following health state scenarios:

- Home recovering
- Hospital with no complications
- Hospital with pneumonia

Full details of how the utility values were calculated using these health state scenarios can be found in the appendix E.

Health state	Standard gamble mean (standard deviation)
Own current health	0.97 (0.06)
Home, disease free	0.96 (0.07)
Home, recovering	0.92 (0.15)
Hospital, no complications	0.90 (0.15)
Hospital, pneumonia	0.82 (0.25)
Recurrence in gastric tube	0.41 (0.31)
Recurrence in bones	0.35 (0.30)
Irresectable	0.34 (0.31)

Table 3-C: Utility values reported by de Boer et al. (2002)
Utility values reported by de Boer *et al.* using standard gamble technique in patients cured of cancer by oesophagectomy.

3.5.2 Utility of various Barrett's disease states

Four studies that examined quality of life in patients with Barrett's oesophagus were identified (Richards et al. 2003; Hur et al. 2005; Garside et al. 2006; Hur et al. 2006). Of these, only two derived utilities transferable to the health states used in a cost utility analysis (Garside et al. 2006; Hur et al. 2006).

The study by Hur et al., set in the US, enrolled 20 patients with non-dysplastic BM, with a mean age of 64.6 years. Each was given instructions on performing standard gamble, and then presented with various health state scenarios. The utilities ascertained are presented in table 3-D

One can approximate health state scenarios from the study by Hur et al., to the health states in this study, as follows:

- Barrett's oesophagus (no dysphagia) *approximates to* post RFA with Barrett's oesophagus
- Post oesophagectomy with dysphagia *approximates to* Anastomotic stricture post oesophagectomy

Health State	Mean (Median)	Range	Standard Deviation
Actual Patient Health State			
Barrett's oesophagus (no dysplasia)	0.95 (0.98)	0.775–0.995	0.067
Hypothetical Patient Health States			
Post-Oesophagectomy with dysphagia	0.92 (0.96)	0.725–0.995	0.079
Post-PDT (no dysphagia)	0.93 (0.98)	0.550–0.995	0.107
Post-PDT with dysphagia	0.91 (0.96)	0.550–0.995	0.117
Intensive Endoscopic Surveillance	0.90 (0.95)	0.550–0.995	0.138

Table 3-D: Utility values reported in the study by Hur et al. (2006)
Utility values reported by Hur et al. using standard gamble technique in patients with non-dysplastic BM.

An additional source of utilities was identified in the HTA on Barrett's surveillance (Garside et al. 2006). In this study the authors recruited a group of 64 people from the general population (the 'Value of Health Panel') and trained them in carrying out the standard gamble method for preference elicitation. Although some of the scenarios are applicable there are several problems in transferring these values for use in this study. Firstly, they have been derived from patients in full health, which although is in line with NICE methods, means they cannot be compared with the studies by de Boer et al. or Hur et al., where utilities were derived from patients. Secondly, Garside et al. assumes that utility remains constant from non-dysplastic BM to HGD. It has been subsequently demonstrated that utility decreases with worsening dysplasia (Gerson et al. 2007). Thirdly, there appear to be discrepancies in the values used, with utility for a person with asymptomatic cancer of 0.875, higher than post-surgery well (0.863), which in turn is higher than a person without Barrett's (0.8). These values are in contrast to the findings of de Boer et al. and Hur et al.

3.5.3 Quality of life studies in RFA patients

At the time of this study, only one published abstract was identified assessing quality of life in patients with BM with HGD (Shaheen et al. 2008). In this US based study, VAS was used to assess different aspects of health related quality of life in patients both at baseline and 12 months after RFA treatment. Each scale was different in its range. Follow up is not complete, with 41/125 of patients having completed the 12 months follow up. With the limited data available for analysis, those with a complete response

to dysplasia had an improvement in daily quality of life, depression and difficulty sleeping.

It is difficult to draw conclusions from these interim results. Only approximately one-third of patients have been followed up thus far. In addition the authors did not use a validated standardised measure of quality of life, such as the SF-36 or EQ-5D. Their method for measuring preference was the VAS with its inherent problems. Their study was designed to study the effects of a treatment on quality of life, rather than giving a patient preference to a particular health state.

Thus utility for the post RFA states were estimated, partly by using utilities derived from the post-PDT health state scenarios from the work by Hur et al. 2006 (see table 3-D), and additionally using the other known recorded values to provide a framework.

3.6 Costs

The costs used in the model are shown in table 3-E. The majority of costs were estimated from NHS reference costs (Department of Health 2009). This derives costs from nationally collected data from NHS trusts and calculates an average total cost of delivering a treatment episode to a patient. The cost of RFA is not available on this national tariff and thus was estimated using direct and variable costs derived from information obtained from the UK distributor (Synetics Medical Limited, Middlesex, UK). All costs were for 2009/10 and are shown in UK pounds sterling. For the sensitivity analysis all cost were varied by +/- 25%. A full breakdown of the costs may be found in the appendix F.

Cost estimate (£)	Base case†	Reference
Treatment course of RFA	3869	Costs supplied by Synetics Medical Ltd
Additional cost from developing perforation from RFA (excludes oesophagectomy)	2619	
Routine gastroscopy and biopsies	406	NHS reference costs 2009/10 (Department of Health 2009)
Elective Oesophagectomy	6342	
Oesophagectomy with complications	9305	
Oesophageal dilatation	427	
Outpatient follow up per visit	72	
Palliation of untreatable adenocarcinoma	3863	(Garside et al. 2006), NHS reference costs 2009/10 (Department of Health 2009)

Table 3-E: Summary of the costs used in the model.
† For the probabilistic sensitivity analysis a Gamma distribution was used based on the assumption that +/- 25% formed a 95% confidence interval.

3.7 Analysis

3.7.1 Deterministic

To enable thorough interrogation of the model and the input parameters, a one-way sensitivity analysis was undertaken, in which the various parameters in the model are varied throughout the specified range to assess how this impacts upon study results (Drummond et al. 2005). For areas where uncertainties in the literature were identified or to interrogate areas where assumptions had been made, a threshold analysis was undertaken, which allows the critical value of a parameter central to the decision to be identified (Drummond et al. 2005). Where uncertainty in the methodology of the model existed such as in the time period of surveillance post RFA, a scenario analysis was undertaken to explore the effects of increasing surveillance.

3.7.2 Probabilistic

Probabilistic sensitivity analysis allows for all input parameters in a model to be specified as full probability distributions, rather than point estimates, allowing a representation of the uncertainty surrounding their values (Claxton et al. 2005). In the UK, it is a requirement for all cost-effectiveness models submitted to NICE (Claxton et al. 2005). The distributions and parameters used for each variable in the model are shown in tables 3-A and 3-B. For probabilities of an event either occurring or not occurring beta distributions were used, which generate values between 0 and 1 and

hence avoids unfeasible values. As the method of generating utilities used was based on standard gamble, negative values were not possible, and hence the beta distribution is appropriate. Where more than two events are possible, Dirichlet distributions were chosen, which ensures the total value of the probabilities of all events considered sum to 1. Gamma distributions cannot take negative values and have a skewed distribution, which is characteristic of many forms of cost data (Doubilet et al. 1985).

The model was repeatedly run 5000 times using values randomly drawn from these distributions. To illustrate the uncertainty relating to the cost-effectiveness results, a cost-effectiveness acceptability curve was calculated, which estimates the probability that an intervention is more cost-effective than its comparator at different values of QALY (Lothgren and Zethraeus 2000).

CHAPTER 4: Immunohistochemical analysis of 5-lipoxygenase pathway in Barrett's adenocarcinoma

In this chapter the results of the immunohistochemical analysis are presented and discussed. A summary may be found in section 4.1. Group demographics and recruitment are in section 4.2. The results of 5-lipoxygenase (5-LOX) pathway protein expression in tissue samples are presented in section 4.3 and their correlation with white cell subtypes in section 4.4. These results are discussed in section 4.5.

4.1 Summary

Striking increases in the immunoexpression of 5-LOX pathway enzymes responsible for the synthesis of leukotrienes (LT) B_4 and C_4 were seen in the stromal area of oesophageal adenocarcinoma (OA) tissue, with 5-LOX ($p=0.041$), 5-lipoxygenase activating protein (FLAP) ($p=0.038$), leukotriene A_4 hydrolase (LTA_4H) ($p=0.0008$) and leukotriene C_4 synthase (LTC_4S) ($p=0.036$) immunopositive cells being approximately three-, eight-, 37-, and six-fold more numerous respectively compared to squamous tissue. FLAP and LTA_4H immunostaining correlated with elevated neutrophil counts ($p<0.001$). Cell counts in Barrett's metaplasia (BM) were intermediate between squamous and OA tissues, except for LTC_4S^+ cells which were as numerous in BM ($p=0.014$ vs squamous) as in OA. LTC_4S immunostaining was also notably overexpressed within epithelial cells in BM ($p<0.001$) and OA ($p<0.01$) tissue. Key biosynthetic enzymes of the LTB_4 and LTC_4 biosynthetic pathways are incrementally expressed across the spectrum of squamous, BM and OA tissues, suggesting a possible role of these leukotriene sub-families in disease progression.

4.2 Immunostaining for 5-LOX pathway proteins in oesophageal biopsies

Distinct nucleated cells with perinuclear immunopositive staining for each of the 5-LOX pathway proteins (5-LOX, FLAP, LTA_4H and LTC_4S) were observed in the stromal areas of oesophageal biopsies.

4.2.1 5-LOX expression

Geometric mean (GM) cell counts per mm^2 of stromal area progressively increased through squamous (GM cell count; GM-SEM, GM+SEM: 8.04; 5.43, 11.9), BM (16.8; 13.3, 21.2), and OA tissue (26.6; 19.8, 35.7) (see figure 4-A). Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA tissue when compared to squamous tissues (p=0.041), but not between BM and squamous (p=0.152), and OA and BM tissue (p=0.242). Representative photomicrographs of stromal staining in OA tissue may be found in figure 4-F.

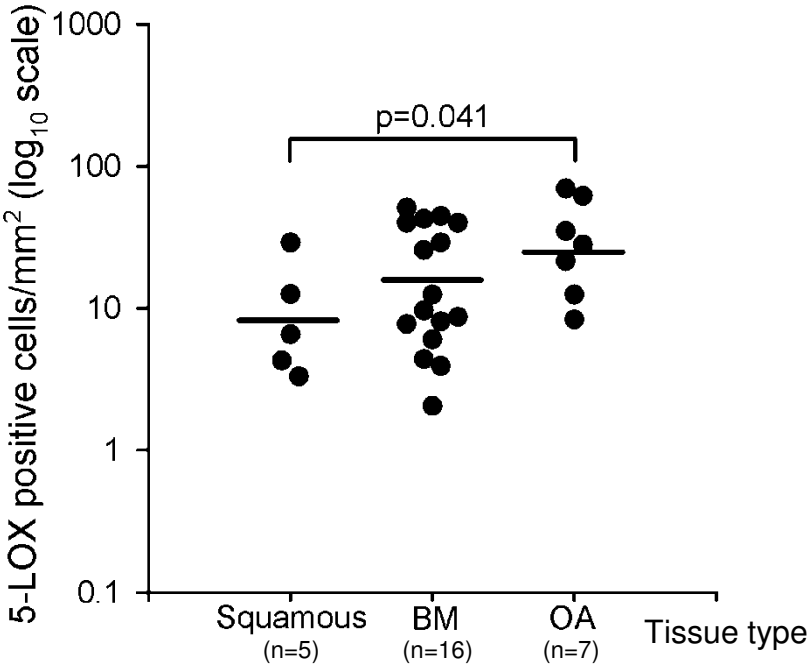


Figure 4-A: Stromal 5-LOX positive cell count in different tissue types.
Counts of cells per mm^2 of stromal area that were immunopositive for 5-LOX are shown (log_{10} Y-axis). GM cell counts (indicated by cross-hair) were significantly higher in OA tissue when compared to squamous tissues (unpaired Student t-test, p values shown).

4.2.2 FLAP expression

FLAP$^+$ GM cell counts per mm^2 of stromal area progressively increased through squamous (7.31; 3.64, 14.7), BM (15.6; 12.4, 19.6), and OA tissue (57.9; 38.5, 87.0) (see figure 4-B). Statistical comparisons between tissue types were made between $\log_{10}$-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA tissue when compared to squamous tissues (p=0.038), and between OA and BM tissues (p=0.019), but not between BM and squamous (p=0.350). Representative photomicrographs of stromal staining in OA tissue may be found in figure 4-F.

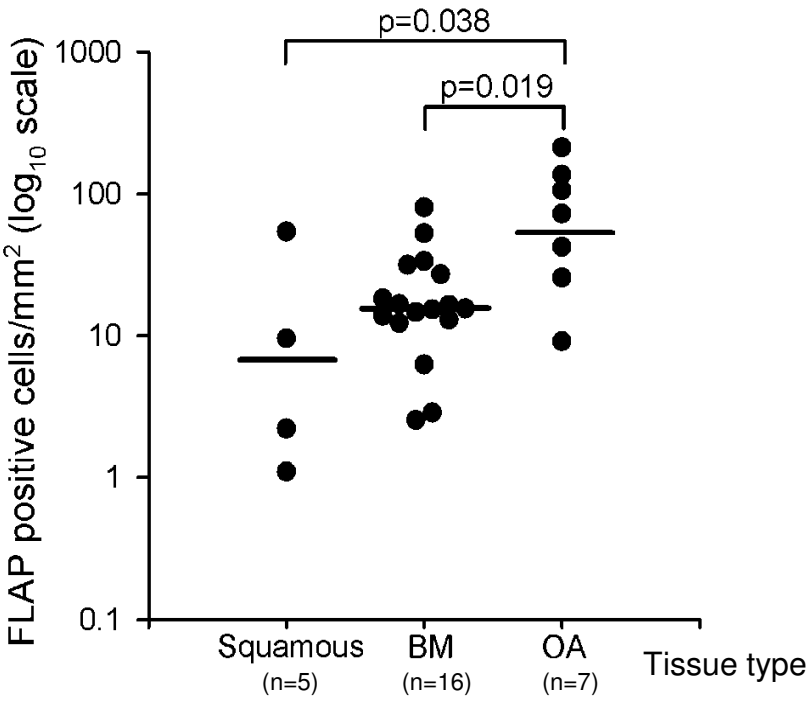


Figure 4-B: Stromal FLAP positive cell count per mm^2 in different tissue types. Counts of cells per mm^2 of stromal area that were immunopositive for FLAP are shown ($\log_{10}$ Y-axis). GM cell counts (indicated by cross-hair) were significantly higher in OA tissue when compared to both squamous and BM tissues (unpaired Student t-test, p values shown).

4.2.3 LTA_4H expression

LTA_4H^+ GM cell counts per mm^2 of stromal area progressively increased through squamous (0.420; 0.313, 0.656), BM (0.878; 0.595, 1.30), and OA tissue (8.90; 5.38, 14.7) (see figure 4-C). Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA tissue when compared to squamous tissues (p=0.0008), and between OA and BM tissues (p=0.002), but not between BM and squamous (p=0.109). Representative photomicrographs of stromal staining in OA tissue may be found in figure 4-F.

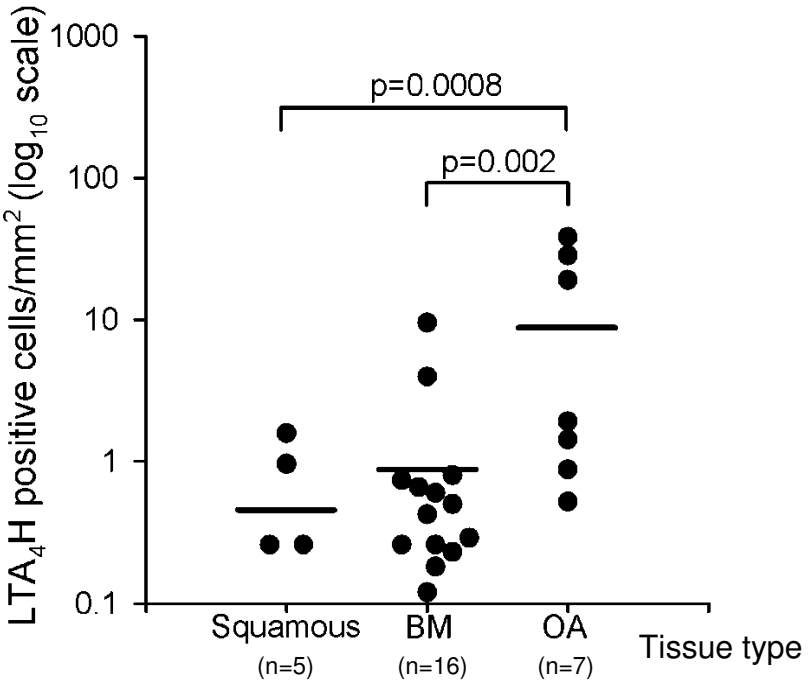


Figure 4-C: Stromal LTA_4H positive cell count in different tissue types
Counts of cells per mm^2 of stromal area that were immunopositive for LTA_4H are shown (log_{10} Y-axis). GM cell counts (indicated by cross-hair) were significantly higher in OA tissue when compared to both squamous and BM tissues (unpaired Student t-test, p values shown).

4.2.4 Stromal LTC_4S expression

LTC_4S^+ GM cell counts per mm^2 of stromal area was highest in BM (25.6; 23.2, 28.3), followed by OA (18.9; 15.1, 23.7) and squamous tissue (3.19; 1.77, 5.73) (see figure 4-D). Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in both OA and BM when compared to squamous tissues (p=0.036 and p=0.024 respectively), but not between BM and OA (p=0.232). Representative photomicrographs of stromal staining in OA tissue may be found in figure 4-F.

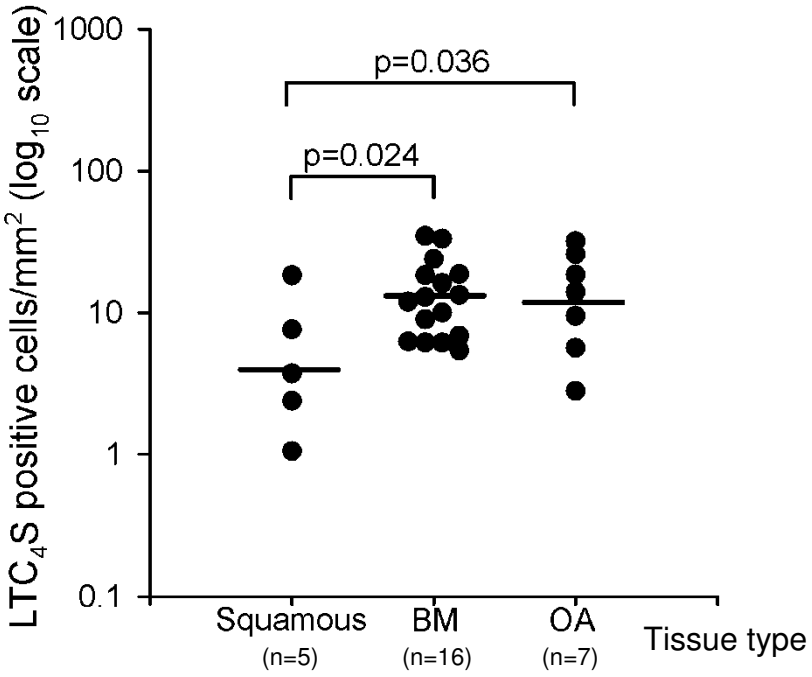


Figure 4-D: Stromal LTC_4S positive cell count in different tissue types.
Counts of cells per mm^2 of stromal area that were immunopositive for LTC_4S are shown (log_{10} Y-axis). GM cell counts (indicated by cross-hair) were significantly higher in both OA and BM when compared to squamous tissues (unpaired Student t-test, p values shown).

4.2.5 Epithelial immunostaining of LTC4S

Epithelial cells in both BM and OA tissue showed immunostaining for LTC_4S that was more cytoplasmic than perinuclear. Minimal staining was evident in squamous epithelium and controls (see figure 4-E). Quantification of percentage epithelial area staining by image analysis demonstrated mean percentage stain (±standard deviation) of 2.57 ± 0.43 in squamous, 9.06±4.08 in BM, and 8.93±5.61 in OA tissues. Data were distributed parametrically and thus statistical comparisons were made between tissue types using unpaired Student t-tests. There was a statistically significant mean difference in percentage epithelial area staining for LTC_4S between BM and squamous tissues (p=0.0000003) and between OA and squamous (p=0.0024), but not between BM and OA (p=0.958). Representative photomicrographs of epithelial staining in BM and OA tissue may be found in figure 4-G.

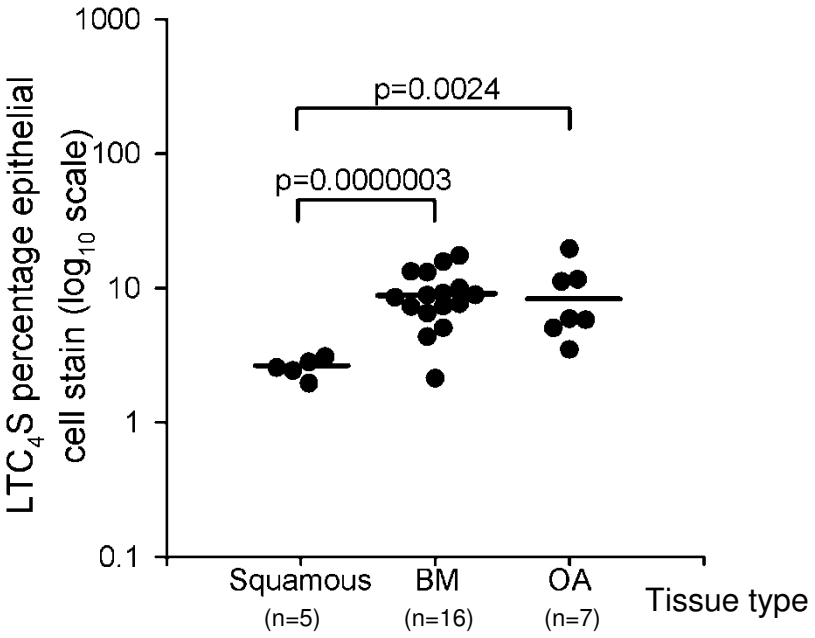


Figure 4-E: Epithelial immunostaining for LTC_4S in different tissue types.
Epithelial LTC_4S immunostaining expressed as a percentage epithelial cell stain (log_{10} Y-axis). Geometric mean value indicated by cross-hair and statistical comparisons between groups were by unpaired student t-test (p values shown).

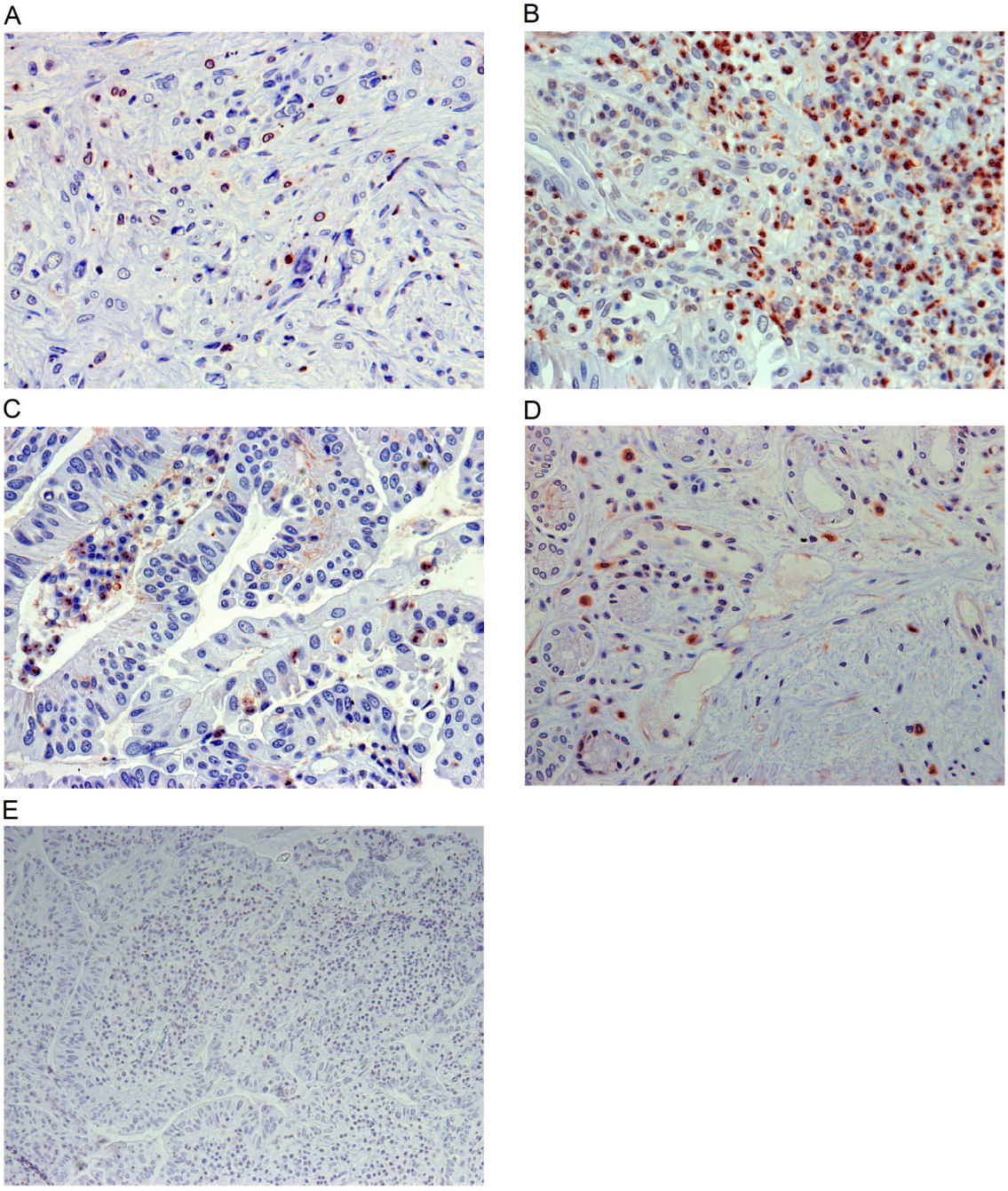


Figure 4-F: Immunostaining for 5-LOX pathway enzymes in stromal area of oesophageal adenocarcinoma tissue.

Representative photomicrographs (x200 magnification) showing cells in stromal area of oesophageal adenocarcinoma tissue immunopositive (red stain) for (A) 5-LOX, (B) FLAP, (C) leukotriene A_4 hydrolase and (D) leukotriene C_4 synthase. Panel E is the control (no primary antibody at x100 magnification).

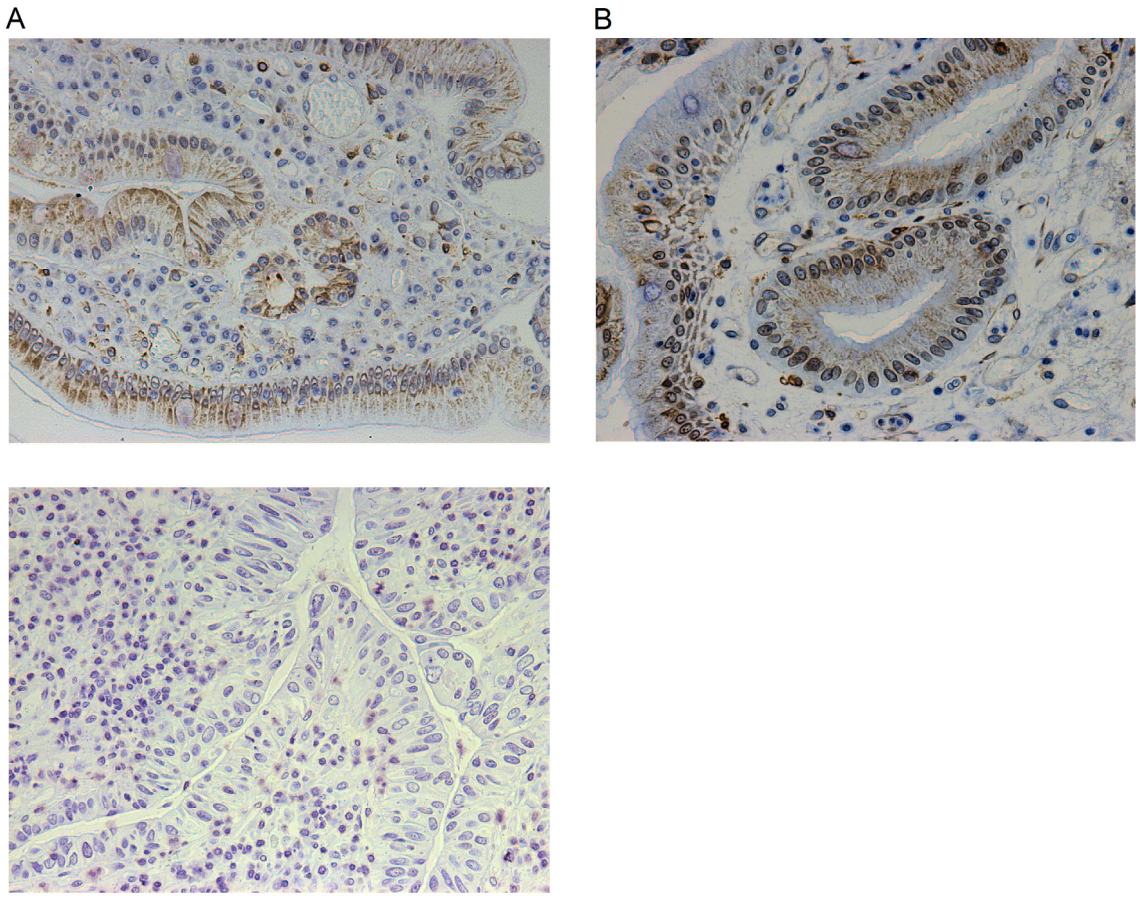


Figure 4-G: Immunostaining for LTC_4 synthase in epithelial area of tissue. Representative photomicrographs (x200 magnification) showing strong epithelial immunostaining (brown stain) for LTC_4 synthase is shown in Barrett's metaplasia (A) and oesophageal adenocarcinoma tissue (B). Panel C is the control (no primary antibody).

4.3 Leukocyte subtype counts and correlation with 5-LOX pathway proteins

To investigate the counts of leukocyte subtypes in tissue types and to correlate these with proteins of the 5-LOX pathway, immunohistochemistry was performed using primary antibodies directed against the following cell-specific markers: neutrophil elastase (NE), CD68 (monocytes/macrophages marker), EG2 antibody to eosinophil cationic protein (eosinophil marker), and CD3 (T-lymphocyte marker). The results are summarised in figure 4-H.

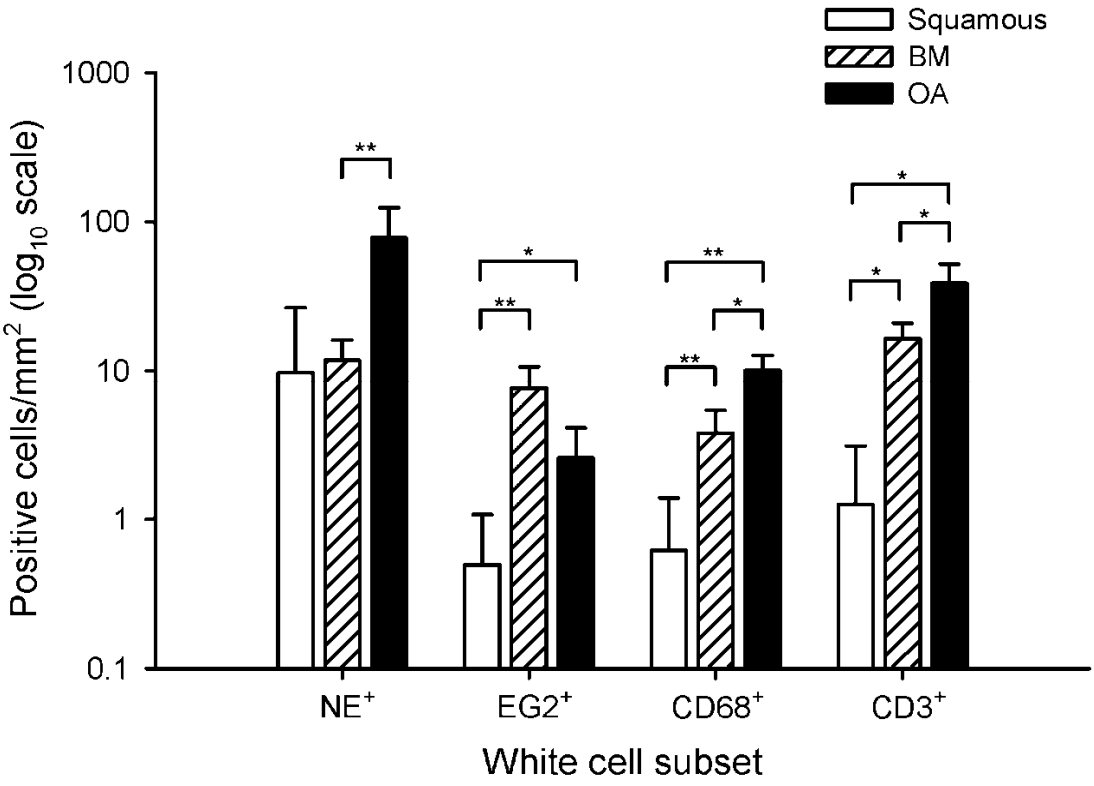


Figure 4-H: Leukocyte counts in squamous tissue, Barrett's metaplasia and oesophageal adenocarcinoma.

Counts of cells immunopositive for neutrophil elastase (NE^+), eosinophil cationic protein ($EG2^+$), monocyte-macrophages ($CD68^+$) and T-lymphocytes ($CD3^+$) are shown (log_{10} Y-axis) in squamous, BM and OA tissue. Histogram bars show geometric mean values (with standard errors) and statistical comparisons between groups were by unpaired Student t-tests with *$p<0.05$ and **$p<0.01$.

4.3.1 NE^+ neutrophils

GM cell counts per mm^2 of stromal area were highest in OA (78.4; 49.1, 125) when compared to both squamous (9.69; 3.54, 26.6), and BM tissue (11.8; 8,62, 16.1). Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA when compared to BM tissues ($p=0.0055$), but not when compared with squamous tissue ($p=0.109$), or between squamous and BM tissue ($p=0.861$).

Regression analyses showed that across all tissues ($n=28$) the log_{10} counts of $FLAP^+$ and LTA_4H^+ cells correlated strongly with neutrophils ($r=0.832$ and $r=0.736$ respectively, both $p<0.001$) (see 4-I).

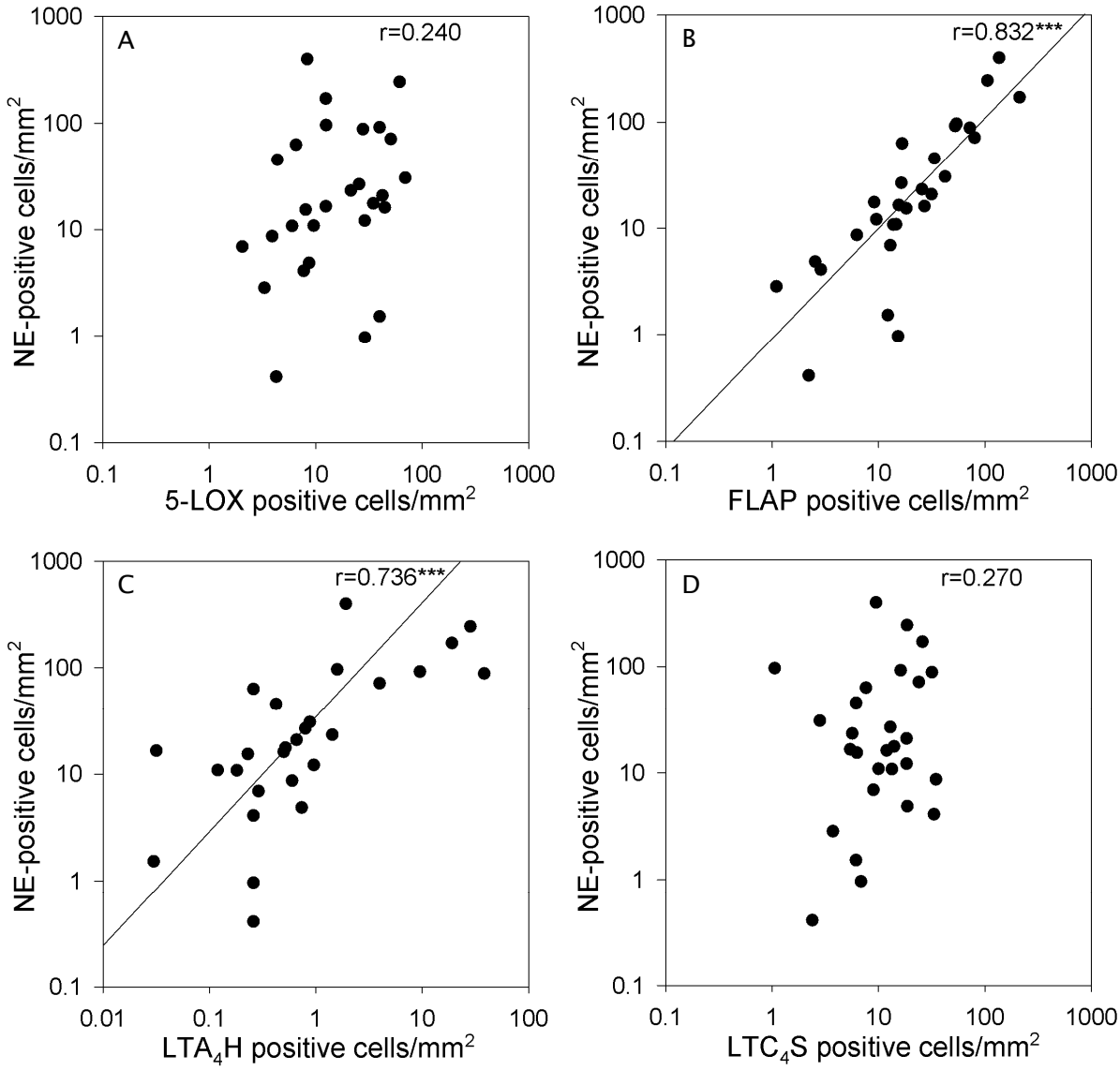


Figure 4-I: Correlation of 5-LOX pathway protein immunoexpression with neutrophil counts.

Scatter plots with line of best fit showing correlations between the counts (per mm^2) of neutrophils (NE^+, Y-axes) with counts of cells immunopositive for (A) 5-LOX, (B) FLAP, (C) LTA_4H and (D) LTC_4S in squamous (n=5), BM (n=16) and OA (n=7) tissues. Pearson's correlation coefficients (r) are shown, with ***p<0.001 for the significant correlations between neutrophils and FLAP and between neutrophils and LTA_4 hydrolase positive cell counts.

4.3.2 CD68 positive monocyte/macrophages

$CD68^+$ GM cell counts per mm^2 of stromal area progressively increased through squamous (0.435; 0.265, 0.714), BM (3.78; 2.67, 5.37), and OA tissue (10.1; 7.96, 12.7) Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA when compared to both BM ($p=0.0306$) and squamous ($p=0.00336$), and between squamous and BM tissue ($p=0.00967$).

Regression analyses showed that across all tissues ($n=28$) the log_{10} counts of $5\text{-}LO^+$ and $FLAP^+$ correlated weakly with monocyte/macrophages ($r=0.468$ and $r=0.452$ respectively, both $p<0.05$) (see figure 4-J).

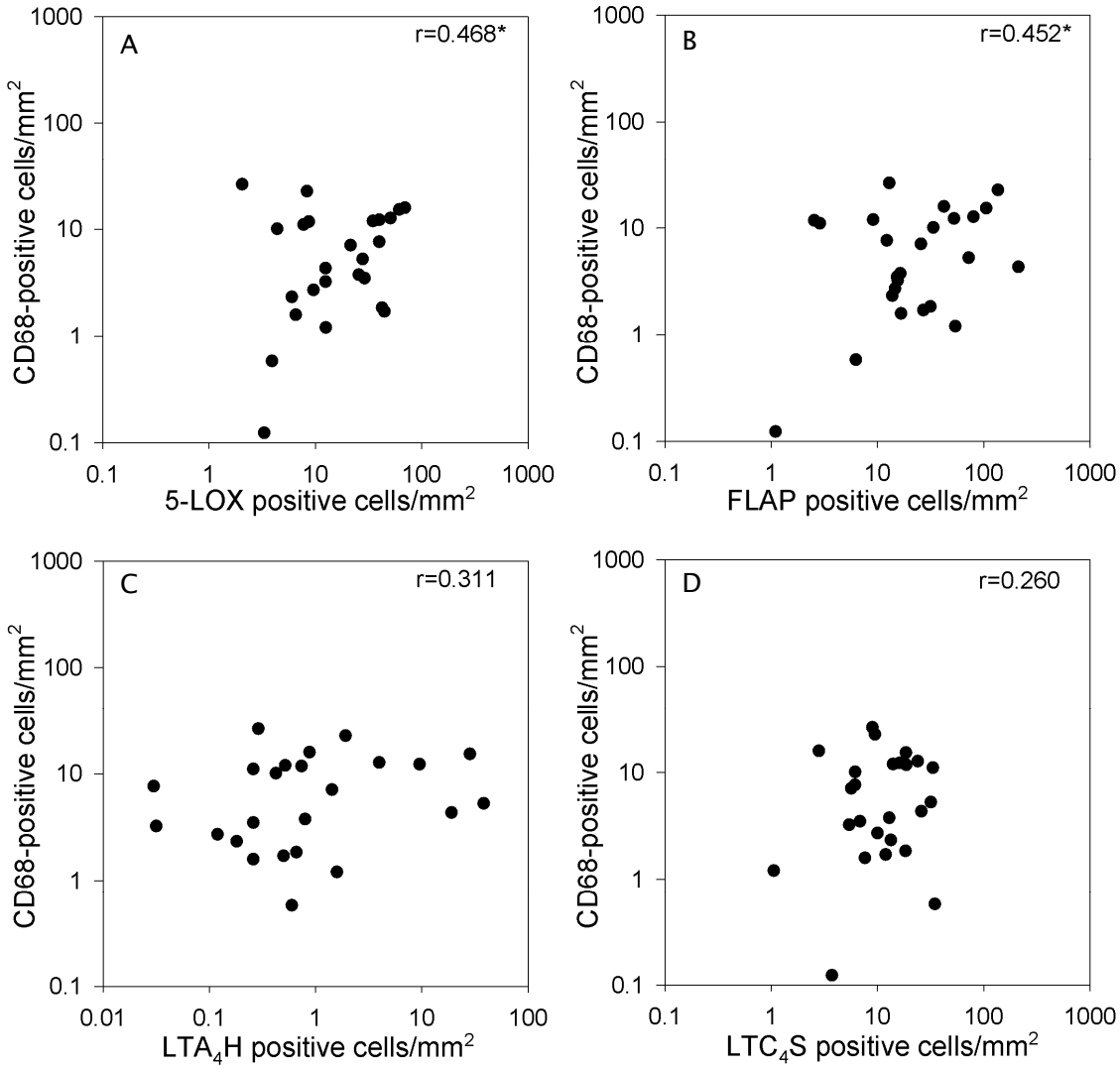


Figure 4-J: Correlation of 5-LOX pathway protein immunoexpression with monocyte/macrophage counts.

Scatter plots with line of best fit showing correlations between the counts (per mm^2) of monocyte/macrophages ($CD68^+$, Y-axes) with counts of cells immunopositive for (A) 5-LOX, (B) FLAP, (C) LTA_4H, and (D) LTC_4S in squamous (n=5), BM (n=16) and OA (n=7) tissues. Pearson's correlation coefficients (r) are shown, with *$p<0.05$ for the significant correlations between CD68 and 5-LOX and between CD68 and FLAP positive cell counts.

4.3.3 EG2 positive eosinophils

$EG2^+$ GM cell counts per mm^2 of stromal area was highest in BM (7.59; 5.40, 10.7), and then OA (2.58; 1.62, 4.10) and squamous tissue (0.439; 0.230, 1.07). Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA and BM when compared with squamous tissue (p=0.0439 and 0.00695 respectively). No significant difference was seen between BM and OA tissues (p=0.0822).

Regression analyses showed that across all tissues (n=28) the log_{10} counts of $5\text{-}LO^+$ correlated weakly with eosinophils (r=0.475, $p<0.05$) (see figure 4-K).

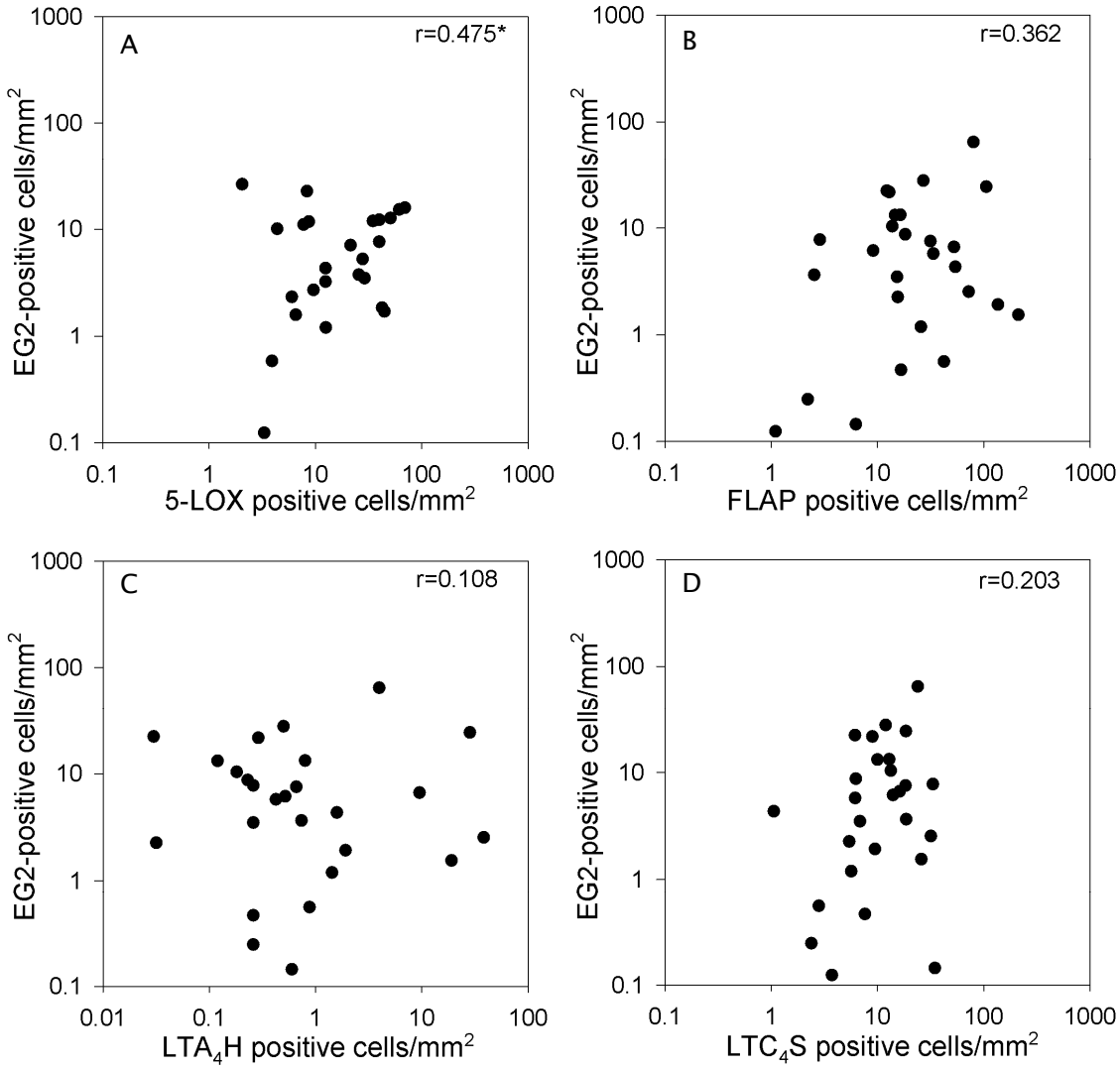


Figure 4-K: Correlation of 5-LOX pathway protein immunoexpression with eosinophil counts.

Scatter plots with line of best fit showing correlations between the counts (per mm^2) of eosinophils ($EG2^+$, Y-axes) with counts of cells immunopositive for (A) 5-LOX, (B) FLAP, (C) LTA_4H, and (D) LTC_4S in squamous (n=5), BM (n=16) and OA (n=7) tissues. Pearson's correlation coefficients (r) are shown, with *$p<0.05$ for the significant correlations between EG2 and 5-LOX positive cell counts.

4.3.4 T-lymphocytes ($CD3^+$)

$CD3^+$ GM cell counts per mm^2 of stromal area progressively increased through squamous (1.27; 0.520, 3.12), BM (16.9; 13.5, 21.2), and OA tissue (38.8; 28.8, 52.2) Statistical comparisons between tissue types were made between log_{10}-transformed values using unpaired Student t-test. GM cell counts were significantly higher in OA when compared to both BM ($p=0.0440$) and squamous ($p=0.0153$), and between squamous and BM tissue ($p=0.0381$).

Regression analyses showed that across all tissues ($n=28$) the log_{10} counts of $FLAP^+$ and LTA_4H^+ correlated weakly with T-lymphocytes ($r=0.507$, $p<0.01$ and $r=0.492$, $p<0.05$) (see figure 4-L).

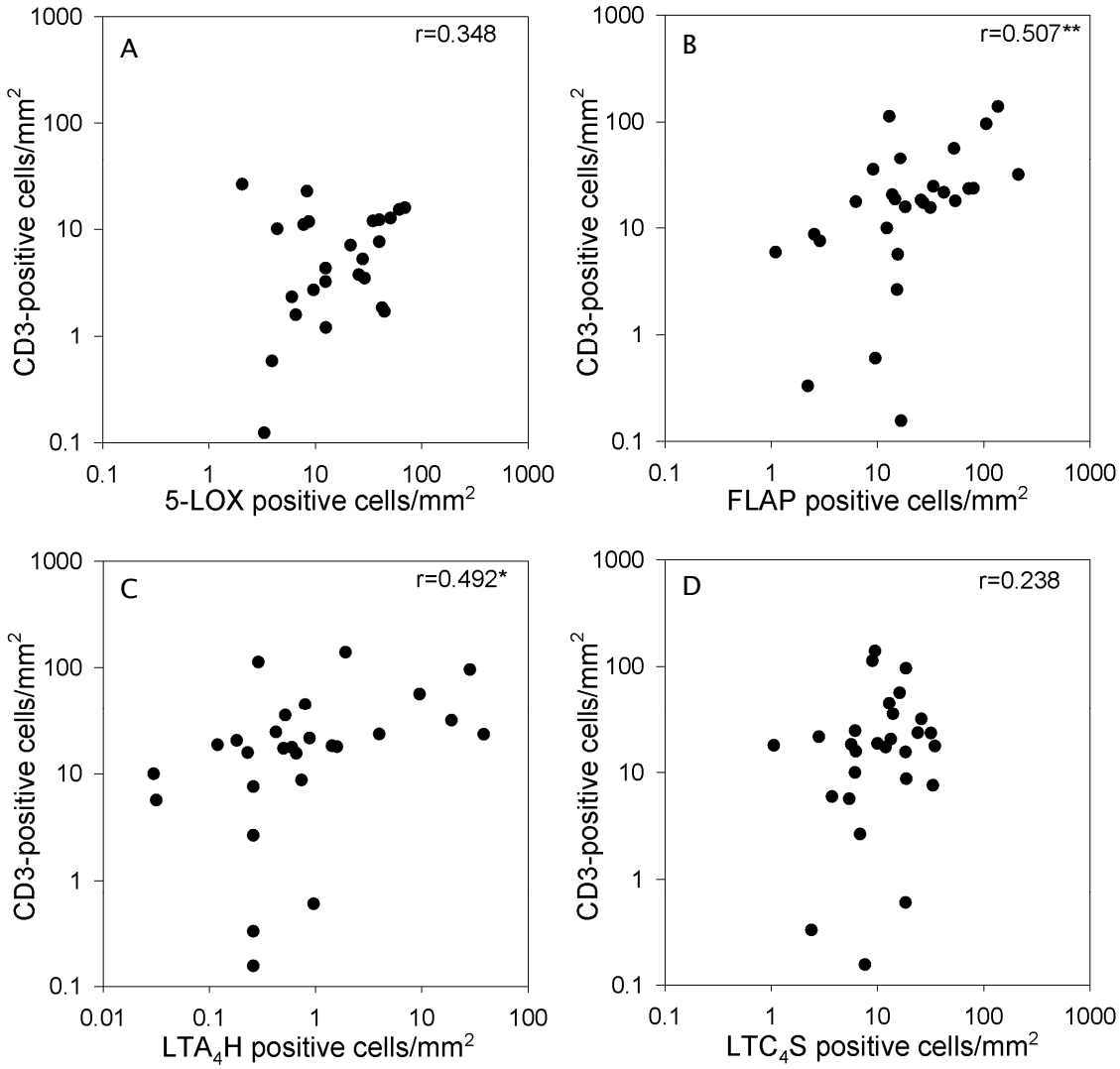


Figure 4-L: Correlation of 5-LOX pathway protein immunoexpression with T-lymphocyte counts.

Scatter plots with line of best fit showing correlations between the counts (per mm^2) of T-lymphocytes ($CD3^+$, Y-axes) with counts of cells immunopositive for (A)5-LOX, (B) FLAP, (C) LTA_4H, and (D) LTC_4S in squamous (n=5), BM (n=16) and OA (n=7) tissues. Pearson's correlation coefficients (r) are shown, with significant correlations between T-lymphocytes and FLAP (**$p<0.01$) and T-lymphocytes and LTA_4H (*$p<0.05$) positive cell counts.

4.4 Recruitment and group demographics

Forty-four patients were recruited, although a proportion of tissue samples were not suitable for analysis. There was difficulty obtaining squamous control tissue with adequate stromal area, with 5 out of 15 biopsies considered of suitable standard for study inclusion. A number of tissue samples suspected to be OA were excluded due to the finding of junctional tumours (3), undifferentiated carcinoma (1), and lack of OA tissue in the sample taken (2).

Characteristics of the patients donating the samples included in the study are shown in table 4-M. There were no significant differences between the groups in age, sex ratio, aspirin or PPI use. Patients in the Barrett's group tended to be younger, whilst those in the OA group used PPI's less than those with BM. There were no aspirin users in any group.

	Squamous	Barrett's	OA	p value
Age	74.7	64.9	77.8	ANOVA=0.124
% male	100	87.5	100	Chi-square=0.647
Aspirin use	0	0	0	-
PPI use	40	93.8	14.3	Chi-square=0.096

Figure 4-M: Patient characteristics

4.4.1 Length of Barrett's segment

Sixteen patients with Barrett's were included for sub-analysis. Segment length exhibited a normal distribution with a mean of 5.9 cm +/- 2.2 cm, median of 6 cm, and a mode of 7 cm (see figure 4-N). Regression analysis revealed no correlation between length of Barrett's segment and cell counts (per mm^2) of 5-LOX, FLAP, LTA_4H, LTC_4S, NE, CD68, EG2, and CD3 (all $r<0.4$, not significant).

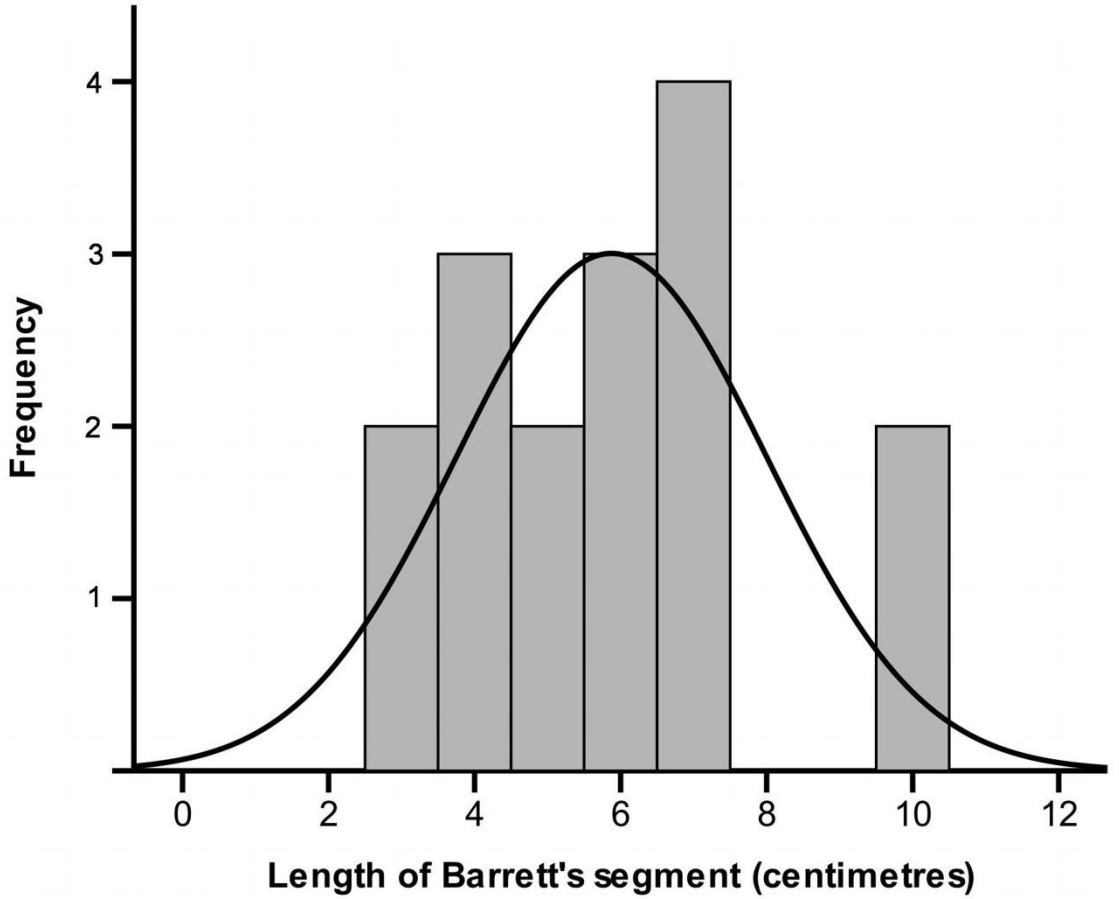


Figure 4-N: Histogram showing frequency and distribution of length of Barrett's segment.

4.4.2 **Proton pump inhibitor (PPI) use**

To analyse the effect of concurrent PPI use at time of tissue acquisition, cell counts were compared between those on PPI (n=19) and those not using PPI (n=6). Data were distributed non-parametrically and thus were compared by Mann-Whitney U test. Mean cell counts per mm^2 were numerically lower in those on PPI for 5-LOX, FLAP, LTA_4H, NE, CD68, EG2, and CD3, although this did not reach statistical significance (see figure 4-O).

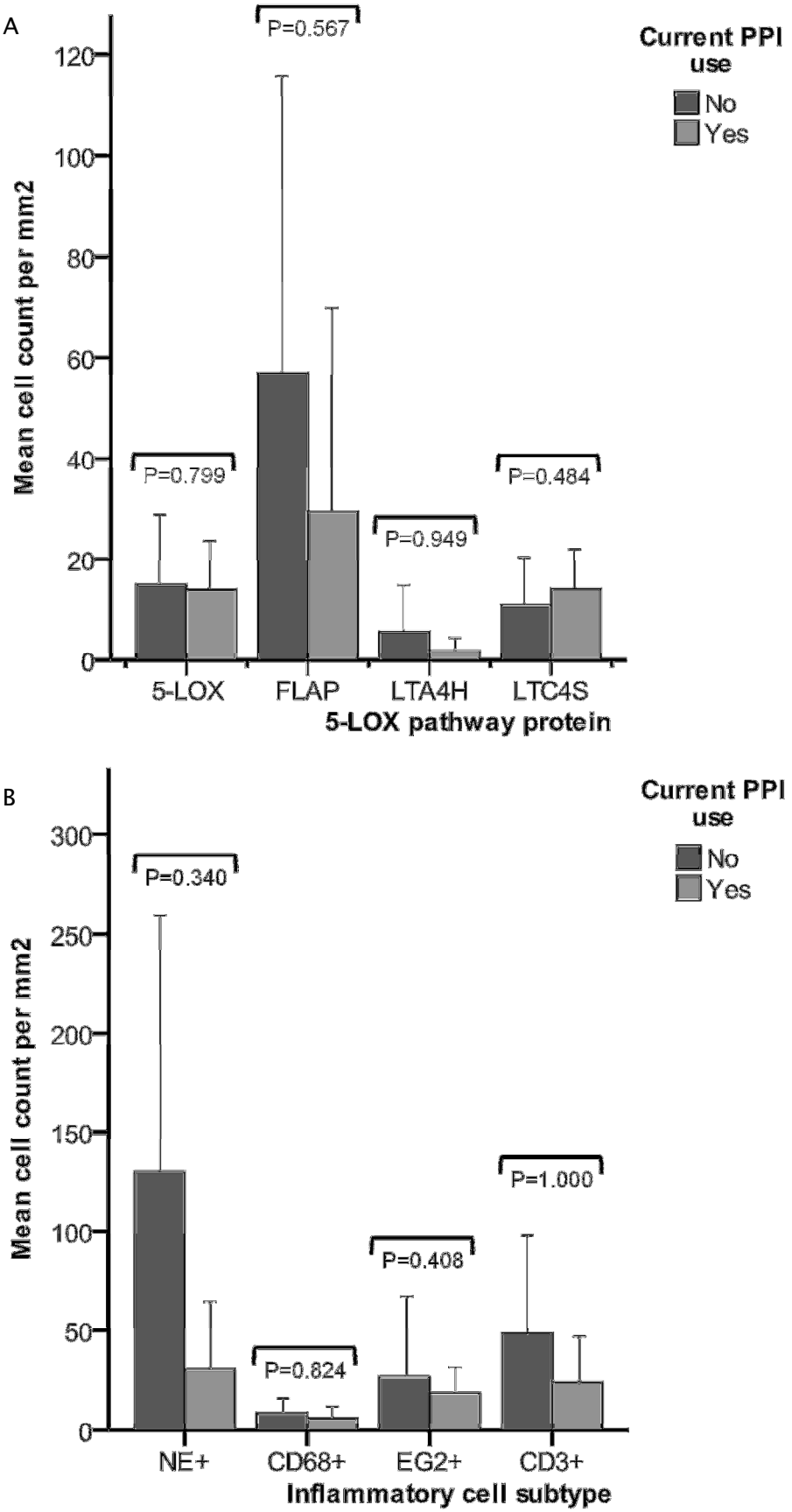


Figure 4-O: Effect of PPI use at time of tissue acquisition on mean cell count per mm^2 of (A) 5-LOX pathway proteins and (B) inflammatory cell subtype.

4.5 Discussion

There is growing recognition that eicosanoid mediators including prostanoids and leukotrienes have roles not only in promoting inflammation but also in carcinogenesis, including cancers of the gastrointestinal tract. Previous studies have identified increased expression and activity of 5-LOX in patients with OA, in animal models of OA, and in OA cell lines (Chen et al. 2004; Hoque et al. 2005). With its activating protein FLAP, 5-LOX supplies an unstable substrate (LTA_4) for the synthesis of the short-lived, receptor-active lipid mediators LTB_4 and LTC_4 by the activities of LTA_4H and LTC_4S respectively. This study has demonstrated firstly that all four of these enzymes are significantly over-expressed in oesophageal biopsies from OA patients compared to squamous control tissue. Secondly, LTC_4S is upregulated in premalignant Barrett's tissue, with unexpectedly strong expression in the oesophageal epithelium of both BM and OA disease groups. These results provide a functional basis for reports of increased LTB_4 production in OA (Chen et al. 2002) and also point to an unsuspected role of the cysteinyl-leukotrienes (LTC_4, LTD_4, LTE_4) in Barrett's disease progression.

These results confirm that 5-LOX expression is increased in OA biopsies (Chen 2004), most likely in several infiltrating leukocyte types including macrophages and neutrophils. Also demonstrated for the first time is a proportionately greater increase in FLAP immunoexpression in the same tissues. FLAP is an integral nuclear envelope protein and an essential, cytokine-inducible cofactor for leukotriene synthesis in intact cells (Dixon et al. 1990); it is also a promising target for novel inhibitors of leukotriene synthesis (Ferguson et al. 2007). Its striking overexpression in OA tissues suggests an increased intracellular capacity to donate arachidonate to 5-LOX to initiate leukotriene synthesis.

Subsequent generation of LTB_4 depends on cell-specific expression of the distal enzyme LTA_4H, which was markedly increased in prevalence (37-fold) in OA tissues, as suggested by a previous semi-quantitative study (Chen et al. 2003). Both FLAP and LTA_4H-positive cell numbers correlated significantly with counts of neutrophils (figure 4-I), which were also elevated in OA biopsies (figure 4-H). These results suggest that an activated 5-LOX/FLAP/LTA_4H pathway is expressed within neutrophils, and that its product LTB_4, a highly potent leukocyte chemotaxin via its LTB_4 receptor, BLT1 (Funk 2001), is implicated in recruiting neutrophils to OA tissues. Neutrophil infiltration is observed in both acute and chronic inflammatory states, and also in different cancers throughout the gastrointestinal tract (Peddareddigari et al. 2010). Neutrophils play an active role in the tumour micro-environment, with neutrophil-derived factors promoting genetic mutations and some, including LTB_4, promoting vascular endothelial growth

factor (VEGF) production from tumour cells, contributing to angiogenesis (Ye et al. 2004; Tazzyman et al. 2009).

4.5.1 Linking 5-LOX pathway production with carcinogenesis

5-LOX pathway products are directly linked to carcinogenesis and tumour cell survival in a variety of tissue sites. The marked anti-proliferative effect of leukotriene synthesis inhibitors in breast cancer cell lines is associated with alterations in a number of apoptotic signalling proteins including B-cell lymphoma (Bcl)2 and Bcl2-associated X protein (Avis et al. 2001). In rats, inhibitors of 5-LOX (zileuton) and of LTA_4H (bestatin) both reduce the incidence and tumour burden in OA (Chen et al. 2003; Chen et al. 2004), suggesting a key role of LTB_4 in oesophageal tumorigenesis. Bestatin however, although widely used as an adjuvant in cancer chemotherapy, has additional aminopeptidase inhibitory activity unrelated to inhibition of LTB_4 synthesis. Although suppression of LTB_4 production with a 5-LOX inhibitor induces apoptosis in oesophageal cancer cell lines, a selective antagonist of the LTB_4 receptor BLT1 (U-75302) was less effective (Hoque et al. 2005). This suggests that LTB_4 receptors other than BLT1, such as BLT2, or more probably 5-LOX products other than LTB_4, play additional roles in oesophageal cancer cell survival. Indeed, the cysteinyl-leukotriene LTD_4 causes time- and dose-dependent increases in β-catenin and Bcl-2 in intestinal cell lines, and the associated reduction in cell death correlates with levels of LTD_4 as well as those of LTB_4 (Ohd et al. 2000).

4.5.2 LTC_4S expression

In this context, the novel finding of increased LTC_4S expression in OA biopsies, both in stromal cells and in epithelial cells, is of particular interest. LTC_4S is an integral nuclear membrane protein and the rate-limiting step for the synthesis of LTC_4, the first of the cysteinyl-leukotrienes, which have a range of vascular, anti-apoptotic and pro-inflammatory actions via cysLT1 and cysLT2 receptors (Kanaoka and Boyce 2004; Ago et al. 2007; Peters-Golden and Henderson 2007), as well as activity at purinoceptors, including $P2Y_{12}$, the target of the anti-platelet drug clopidogrel (Paruchuri et al. 2009). LTC_4S has a relatively restricted cellular expression in some myeloid leukocyte sub-types, but has also been described in bronchial epithelial cells (Jame et al. 2007). The expression of LTC_4S in oesophageal epithelial cells is therefore novel and also intriguing on two accounts. Firstly, oesophageal epithelial cells did not express the proximal enzymes 5-LOX or FLAP, suggesting that epithelial cells may generate LTC_4 from its precursor LTA_4 donated by myeloid leukocytes in a process of transcellular synthesis, as proposed in other tissues (Fabre et al. 2002). Secondly, while 5-LOX, FLAP and LTA_4H tended to be more prevalent, only LTC_4S was significantly over-expressed in BM biopsies, as well as in OA biopsies. Confidence in the finding of epithelial LTC_4S is

merited by careful measures to avoid non-specific immunostaining, as shown in the non-immune rabbit serum control. These included the use of the membrane solubiliser Tween-20, the dilution of the primary antibody with bovine serum albumin to prevent Fc receptor binding, and treatment of the tissue with avidin and biotin block to prevent secondary biotinylated antibody binding (Buchwalow and Böcker 2010). The data suggests that stromal and epithelial LTC_4S upregulation may represent a relatively early marker of Barrett's disease progression.

4.5.3 Expression of 5-LOX, FLAP and LTA_4H in BM tissue

Although a significant increases in immunoexpression of 5-LOX, FLAP or LTA_4H in the BM tissues was not found, it is possible that this may vary with the inflammatory gradient along a Barrett's segment, with inflammation maximal at the new squamo-columnar junction and minimal distally (Fitzgerald et al. 2002a). In the study in this thesis, biopsies were taken 2cm from the oesophago-gastric junction for consistency, but given the mean segment length of 5.9cm, this is approximately 4cm distal from the site of maximal inflammation at the squamo-columnar junction. Further work is thus required to map the expression of the 5-LOX pathway along the entire Barrett's segment. It also does not preclude changes in enzymatic activity of the 5-LOX pathway occurring without changes in enzyme expression. The findings of increased levels of LTB_4 in both oesophagitis and BM, with a significant reduction in LTB_4 levels after gastric acid production was suppressed by a PPI (Triadafilopoulos et al. 1996), suggest that gastric acid exposure may modulate leukotriene synthesis enzymatically, perhaps by pH-dependent activation of infiltrating leukocytes. Additional risk factors such as aspirin and other NSAIDs which inhibit COX-1 may promote leukotriene synthesis enzymatically by shunting of arachidonate from the COX to the 5-LOX pathway. Such effects would be magnified by the concurrent over-expression of all four 5-LOX pathway enzymes observed in OA tissues.

4.5.4 Conclusions

Overall, the translation of these laboratory findings into clinical practice will depend on understanding the complex interplay of the eicosanoid products of 5-LOX and related pathways, such as the COX-2 products also implicated in BM and OA (Shirvani et al. 2000; Souza et al. 2000; Buttar et al. 2002; Kaur and Triadafilopoulos 2002; Jankowski and Anderson 2004). Dual inhibition of the 5-LOX and COX-2 pathways may be required for optimum therapy, as suggested by the additive effects of zileuton and the COX-2 inhibitor celecoxib in a rat model of OA (Chen et al. 2004). The likelihood that different eicosanoids have pro- and anti-tumorigenic properties make a better understanding of this microenvironment of paramount importance prior to extended trials of such therapy (Shureiqi and Lippman 2001)

CHAPTER 5: Isolation and extraction of cellular RNA by laser capture micro-dissection, reverse transcription and real-time polymerase chain reaction

In this chapter the results of gene expression of 5-lipoxygenase (5-LOX) pathway enzymes and receptors (BLT1 and cysLT1R) from areas of squamous, Barrett's metaplasia (BM) and oesophageal adenocarcinoma (OA) tissue are presented and discussed. A summary may be found in section 5.1. Protocol validation is found in section 5.2 and then results of gene expression of 5-LOX pathway transcripts in different cellular areas are presented in section 5.3. The results are discussed in section 5.4.

5.1 Summary

Retrieval of mRNA from laser capture cells using a filter based method was superior to using TRIzol reagent (p=0.009). All gene expression assays performed well with acceptable efficiencies, error and melt curve analysis. The pairing of the housekeeping genes *18s* with *ACTB* performed with the least variability over tissue types. *PTPRC* gene was expressed at very low levels suggesting minimal contamination of laser captured epithelial cell populations with white cells.

Transcript levels for all 5-LOX pathway enzymes, and in particular *ALOX5* (p=0.045), were higher in stromal compared with epithelial areas of all tissue types. Transcriptional expression was highest in the stromal area of OA tissue for 5-LOX pathway enzymes. mRNA transcripts of the cysteinyl leukotriene (cysLT) (*CYSLT1R)* and leukotriene B_4 (LTB_4) (*LTB4R)* receptor genes were expressed most prominently in Barrett's stromal and OA epithelial areas respectively. These results would suggest transcriptional upregulation, although marked sample to sample variation was found, with some expressing little mRNA. Together with the immunohistochemistry results, this would suggest an important role for post transcriptional factors of 5-LOX pathway protein translation in the stroma of OA tissue. Additionally, expression of *LTB4R* by epithelial cells of OA tissue, suggest a functional role for LTB_4 acting on BLT1 receptors in carcinogenesis.

5.2 RNA extraction and Validation of Laser capture micro-dissection

5.2.1 Comparison of TRIzol® and column based method for the extraction of mRNA from laser captured samples

Five paired samples (1 squamous, 2 BM, and 2 OA) were cut by laser capture microdissection and mRNA extracted using one of two methods (as described in methods section 2.3.3.2); filter based using the RNAqueous-Micro kit (Ambion) or using TRIzol® regent. The quantity of mRNA in the samples was assessed using the Nano-drop spectrophotometer.

Mean mRNA content was 11.8+/-9.79ng/µl for the filter based method and 7.07+/-8.24ng/µl for the TRIzol® based method. Samples were paired and data distributed parametrically and thus paired-samples t-tests were employed for statistical analyses. The mean difference in mRNA content was 4.76ng/µl (95% CI: 1.97, 7.56). There was a statistically significant difference in the mRNA content between filter-based and TRIzol® based methods (p=0.009) (see figure 5-A). The filter based RNAqueous Micro Kit was therefore incorporated as standard in the isolation of mRNA from laser captured samples.

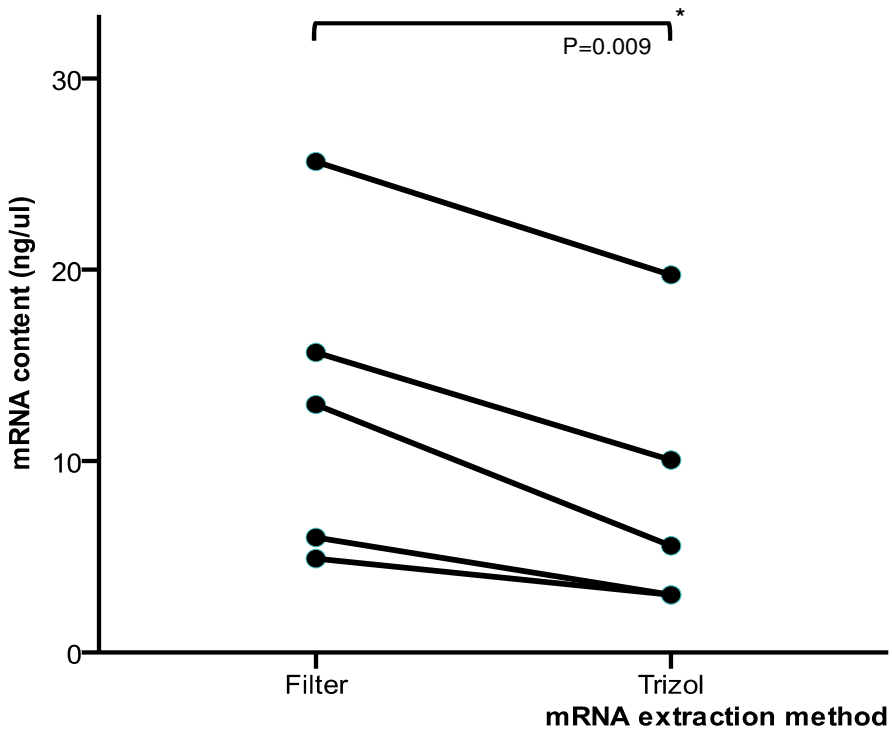


Figure 5-A: Comparison of filter based and TRIzol® reagent based extraction methods from laser captured material.
Quantity of mRNA (ng/µl) extracted from samples using filter based or TRIzol® reagent is shown (Y-axis). The filter based method was superior for the extraction of mRNA in the laser captured samples (* statistical analysis using paired sample student t-test).

5.2.2 Validation of gene expression assays

All gene expression assays used in the real time quantitative polymerase chain reaction (RT-qPCR) experiments were validated as described previously (see section 2.3.4.3). All gene expression assays and primers performed well with acceptable efficiencies between 1.9 and 2.2, and errors of less than 0.2 (table 5-A). An example of amplification and standard curves generated for *ALOX5AP* are shown in figure 5-B. Melt curve analysis was performed on all gene expression assays and primers. All showed just a single peak demonstrating the absence of any other PCR product or primer/dimers. An example of a melt curve for *PTPRC* is shown in figure 5-C.

Gene	Efficiency	Error
ALOX5	2.071	0.020
ALOX5AP	2.185	0.040
LTA4H	2.016	0.002
LTC4S	1.961	0.006
LTB4R	1.941	0.012
CYSLT1R	1.910	0.032
PTPRC	1.922	0.072
ACTB	1.952	0.021
18S	1.911	0.015
GAPDH	1.997	0.003
HPRT1	1.954	0.002

Table 5-A: Efficiency and error of gene expression assays used in real time RT-qPCR experiments.

An efficiency of two represents the perfect PCR reaction, corresponding to doubling of target nucleic acid for each cycle. The error is a measure of the accuracy of the standard curve for quantification, with an acceptable value of below 0.2.

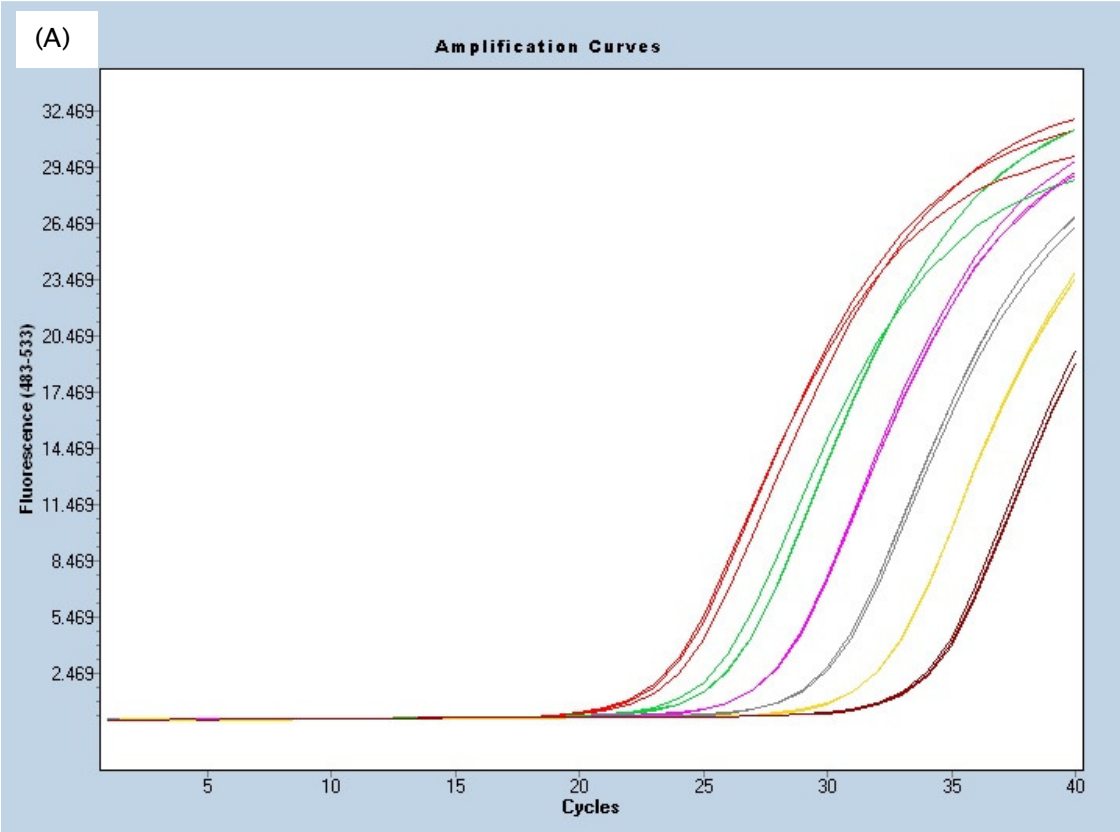
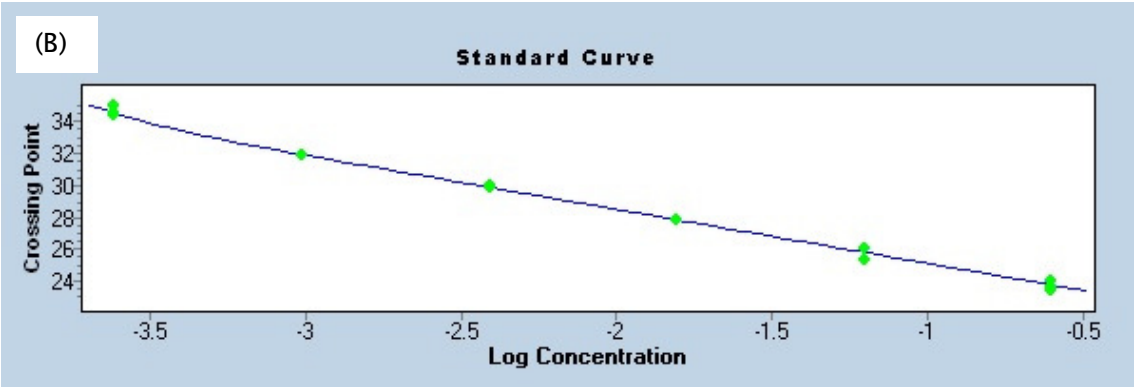


Figure 5-B: Amplification and standard curves
Examples of (A) amplification curves for a titration series of granulocytes with *ALOX5AP* and (B) the calculated standard curve (log concentration plotted against the Cp value).



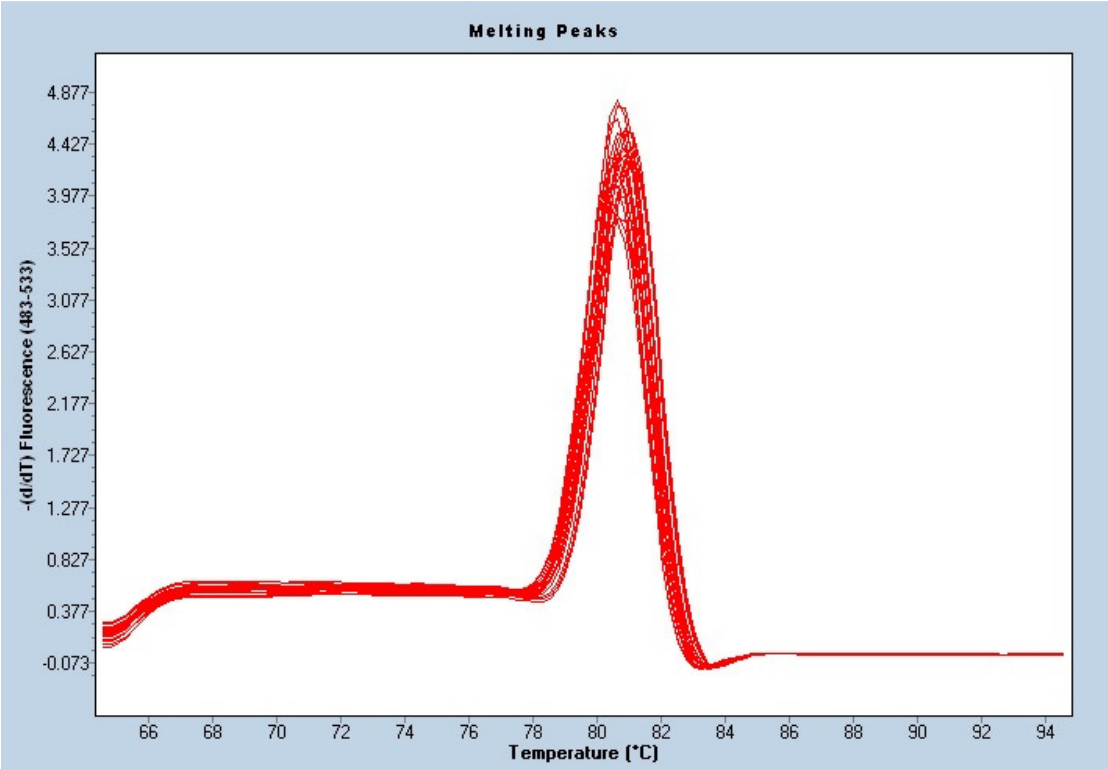


Figure 5-C: Melt curve for *PTPRC*

The PCR product was heated through 65˚C to 95˚C (X-axis), as SYBR green fluorescence was continuously measured (Y-axis). A single peak was seen representing the temperature at which the PCR product binds and separates. Absence of other peaks excludes the presence of primer-dimers and/or nonspecific PCR products.

5.2.3 Assessment of performance of housekeeper genes

The performance of pairs of the four housekeeping genes was assessed in a cross-section of tissue samples (see methods section 2.3.4.3). The pairing of *18s* with *ACTB* performed with the least variability over tissue types (figure 5-D), and was thus chosen as endogenous controls in mRNA expression analysis of the genes of interest in tissue samples. *HPRT1* was a poor housekeeping gene in the tissue samples, with over 50-fold difference in mean expression when combined with *GAPDH*, *ACTB*, or *18s*.

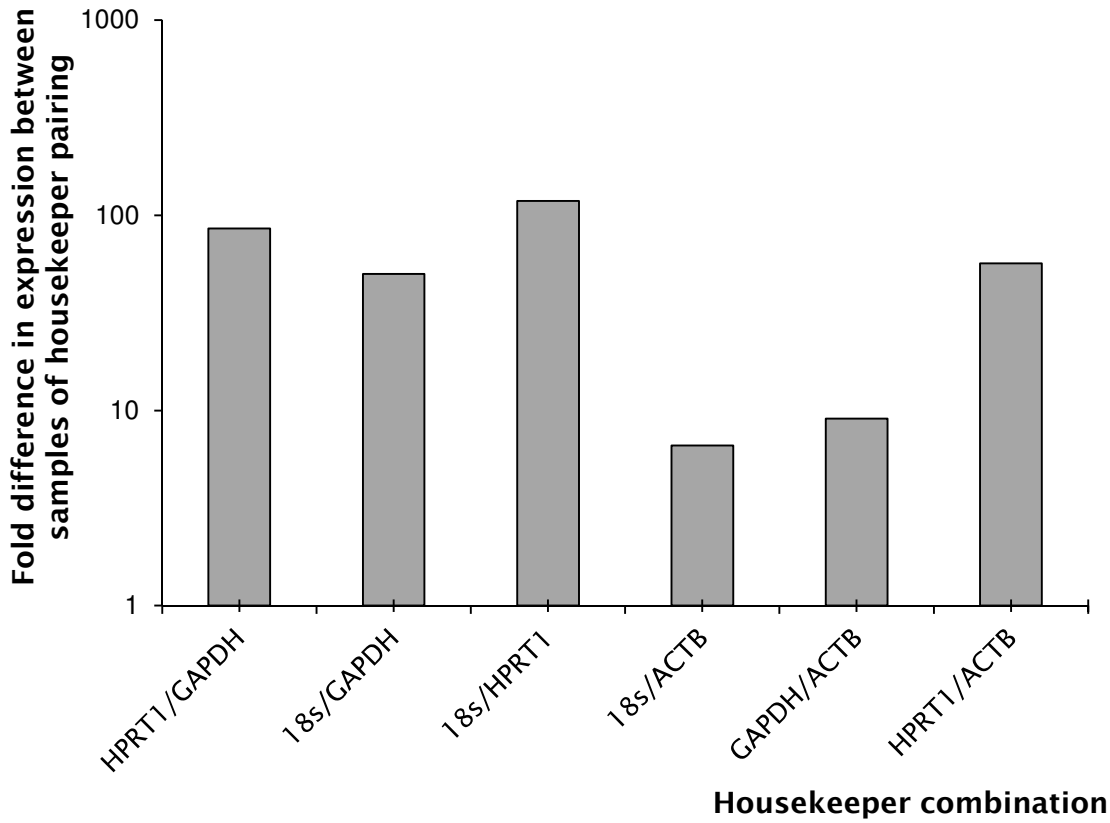


Figure 5-D: Performance of housekeeping genes measured by the ratio of difference in expression between housekeeper pairings.
The pair with the lowest value, i.e. least variability, was chosen for use in the final analysis. The pairing of *18s* and *ACTB* performed best.

5.2.4 Analysis of purity of laser captured epithelial cell populations

The *PTPRC* gene is known to be expressed exclusively in cells of haematopoietic origin with the exception of erythrocytes. To assess the purity of laser captured epithelial tissue areas, the expression of *PTPRC* gene transcripts was assessed. Only very low transcript levels of *PTPRC* gene were found (when compared with granulocyte calibrator), with mean normalised concentration ratios of 0.05+/-0.04 in squamous, 0.06+/-0.06 in BM, and 0.10+/-0.04 in OA tissue (figure 5-E).

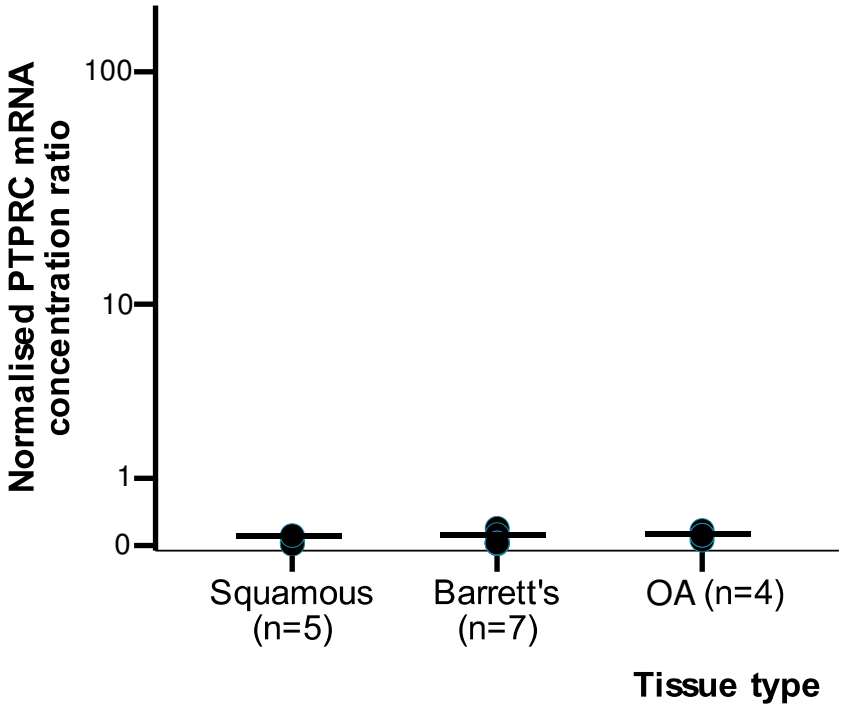


Figure 5-E: *PTPRC* transcript expression in laser captured samples of epithelial cell populations from different tissue types.
Low *PTPRC* gene transcript levels (relative to granulocytes) were found suggesting minimal contamination of laser captured epithelial cell populations with white cells.

5.3 LMD results

5.3.1 Gene expression between different cellular regions

Transcript levels were numerically higher in stromal areas of all tissue types compared with epithelial area (mean difference in normalised mRNA concentration ratios; 95% CI) for *ALOX5* (0.12; 0.00, 0.24), *ALOX5AP* (2.22; -0.45, 4.89), *LTA4H* (8.84; -1.45, 19.13), *LTC4S* (0.74; -0.26, 1.73) and *CYSLT1R* (0.16; -0.14, 0.45) (figure 5-F). Samples were distributed parametrically and thus independent-samples t-tests were employed for statistical analyses. There was a statistically significant difference between stromal and epithelial areas for *ALOX5* gene transcript levels (p=0.045), but not *ALOX5AP* (p=0.099), *LTA4H* (p=0.089), *LTC4S* (p=0.142), nor *CYSLT1R* (p=0.280). Conversely, transcripts of *LTB4R* gene were 7-fold lower in stromal compared with epithelial area with a mean difference of 2.36 (95% CI: -3.54, 8.25), although this did not reach statistical significance (p=0.418).

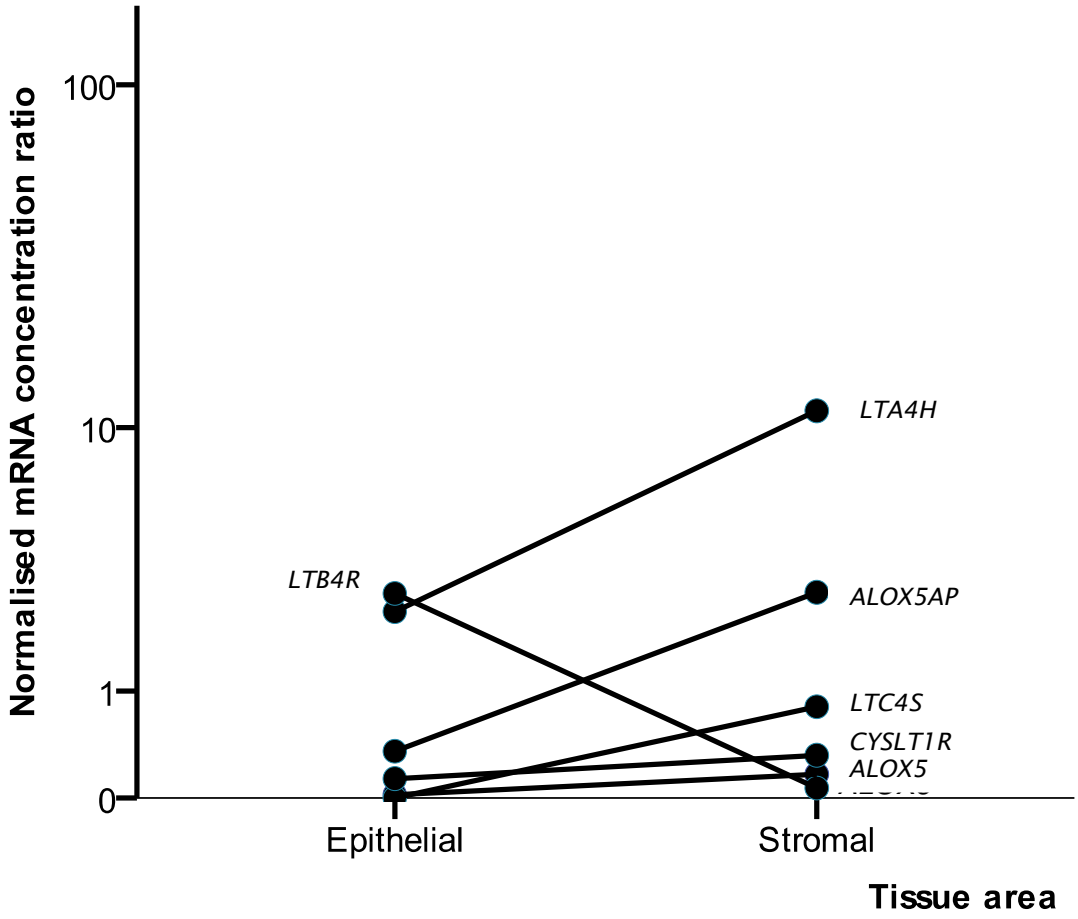


Figure 5-F: mRNA transcript levels of 5-LOX pathway genes of interest in epithelial and stromal areas.

Mean normalised mRNA concentration ratio was higher in stromal areas for the majority of the 5-LOX pathway genes interrogated, with the exception of *LTB4R* which was expressed in higher levels in the epithelial areas of tissue samples.

5.3.2 Transcript expression of 5-LOX pathway genes

5.3.2.1 *ALOX5*

Transcripts of *ALOX5* gene were expressed in only small quantities in all tissue types (figure 5-G). In epithelial areas, mean normalised mRNA concentration ratios (mean+/-SD) were 0.087+/-0.096 in squamous, 0.008+/-0.009 in BM, and 0.015+/-0.021 in OA tissue. The highest levels of *ALOX5* gene transcripts were found in stromal OA tissue: mean normalised mRNA concentration ratios were 0 in squamous, 0.153+/-0.227 in BM, and 0.237+/-0.257 in OA tissue. Data were distributed non-parametrically and thus the Mann-Whitney U test was used for all statistical comparisons between groups. There was no significant difference in *ALOX5* gene transcript levels between different tissue types in either epithelial or stromal areas.

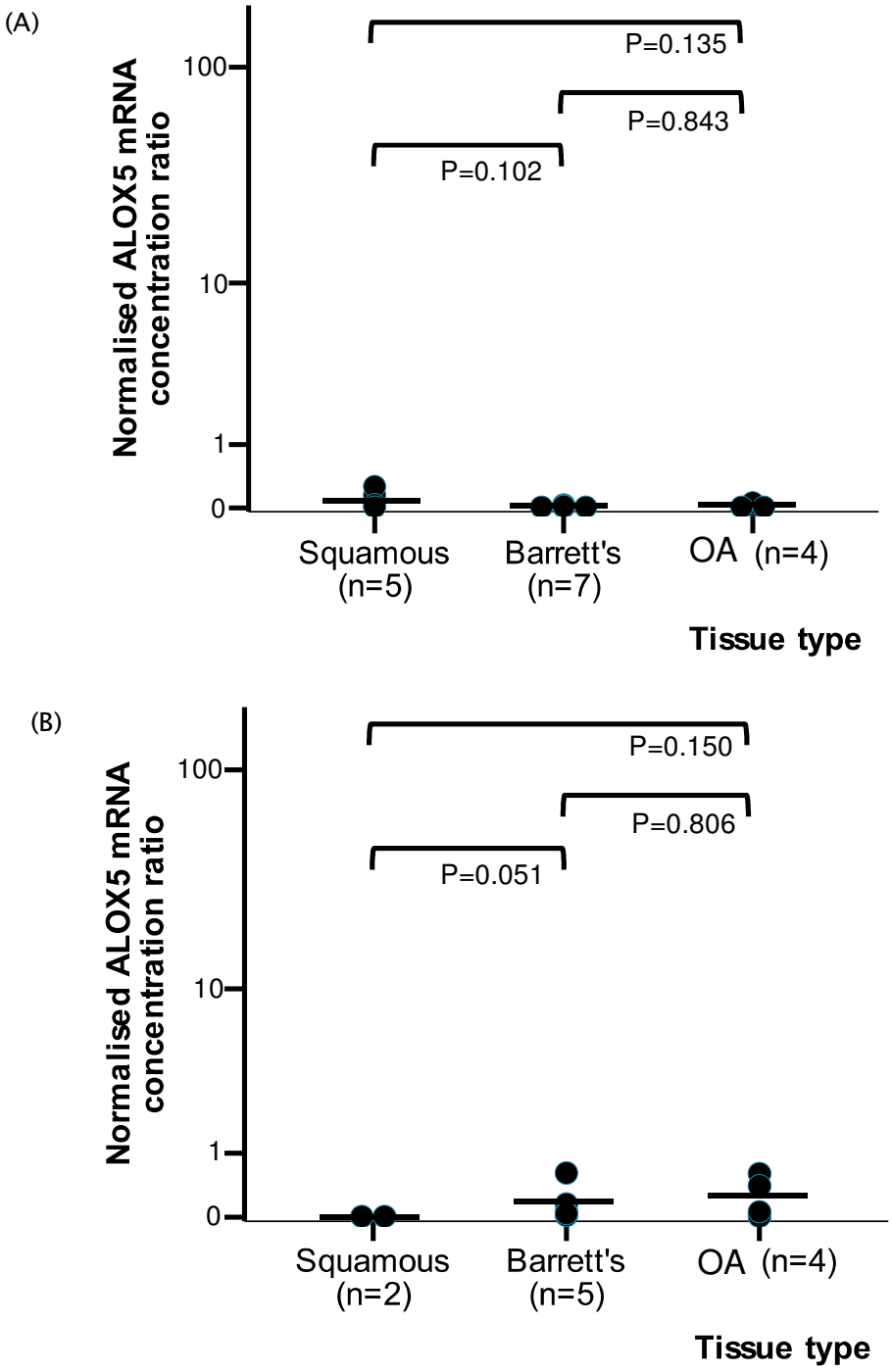


Figure 5-G: *ALOX5* gene transcript levels in (A) Epithelial and (B) Stromal areas
Only low transcript levels of *ALOX5* gene were found in all tissue types. Mean normalised mRNA concentration ratios were highest in the stromal area of OA tissue, although this did not reach statistical significance when compared with either squamous control or Barrett's tissue.

5.3.2.2 *ALOX5AP*

Low levels of *ALOX5AP* gene transcripts were found in the epithelial area of all tissue types, with mean normalised mRNA concentration ratio (mean+/-SD) of 0.18+/-0.21 in squamous, 0.21+/-0.39 in BM, and 0.67+/-0.95 in OA tissue. In contrast, gene transcript levels were 3 to 5-fold higher than granulocyte calibrator in the stromal areas of both squamous and OA tissue, with the highest levels in OA tissue (figure 5-H). Mean normalised mRNA concentration ratios were 3.02+/-4.25 in squamous, 0.07+/-0.11 in BM, and 5.37+/-7.93 in OA tissue. Data were distributed non-parametrically and thus the Mann-Whitney U test was used for all statistical comparisons between groups. There was no significant difference in *ALOX5AP* gene transcript levels between different tissue types in either epithelial or stromal area.

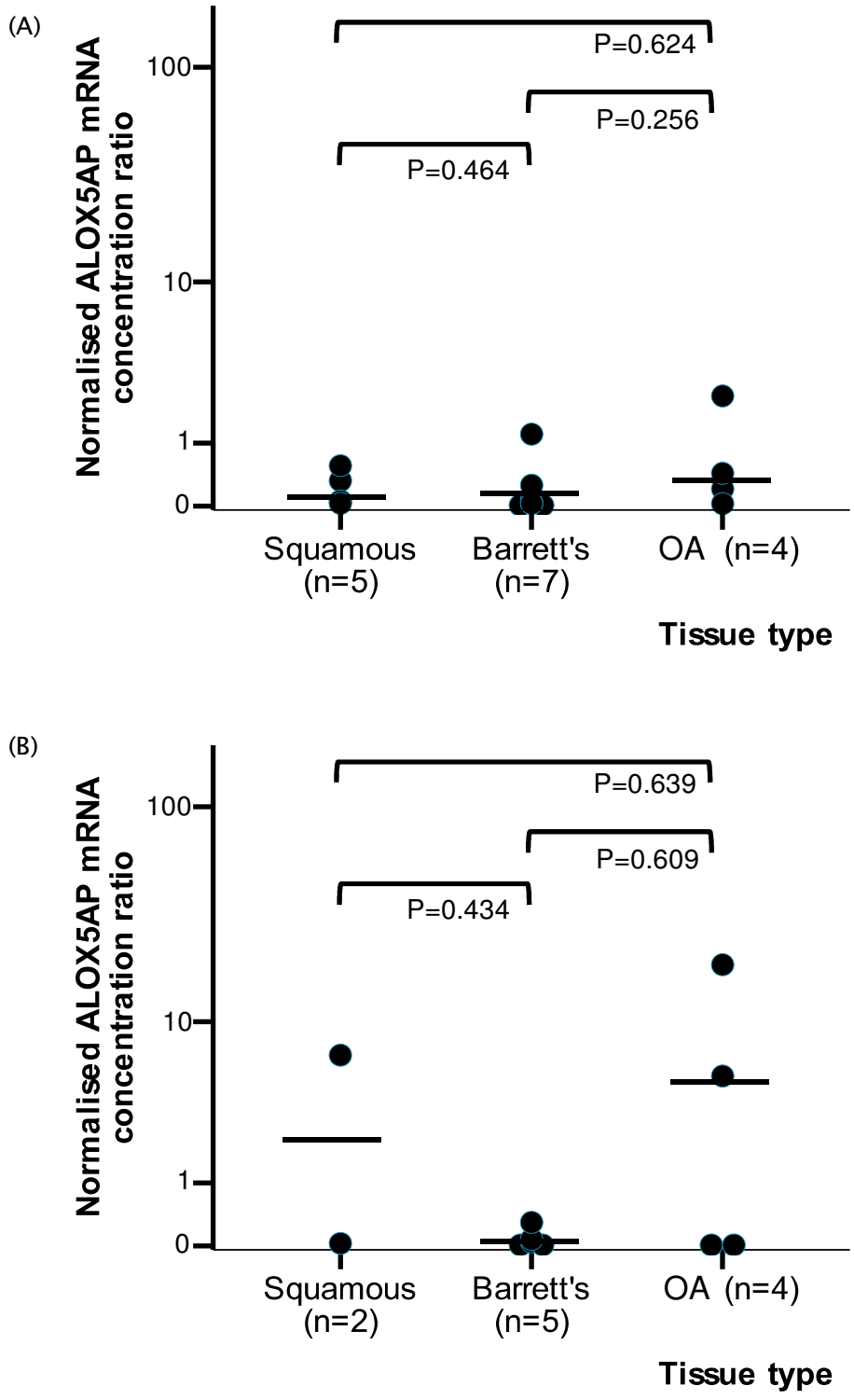


Figure 5-H: *ALOX5AP* gene transcript levels in (A) epithelial and (B) Stromal areas. Gene transcript levels were highest in the stromal area of OA tissue, although this did not reach statistical significance when compared with either squamous control or BM tissue.

5.3.2.3 *LTA4H*

High levels of *LTA4H* gene transcripts were found across all tissue types, with highest levels found in OA tissue (figure 5-I). In epithelial areas, mean normalised concentration ratios (mean+/-SD) were 2.57+/-1.28 in squamous, 1.40+/-1.91 in BM, and 3.75+/-3.22 in OA tissue. In stromal areas, mean normalised concentration ratios were 3.55+/-3.95 in squamous, 4.40+/-3.81 in BM, and 21.97+/-28.98 in OA tissue. Data were distributed non-parametrically and thus the Mann-Whitney U test was used for all statistical comparisons between groups. There was no significant difference in *LTA4H* gene transcript levels in either epithelial or stromal area between different tissue types.

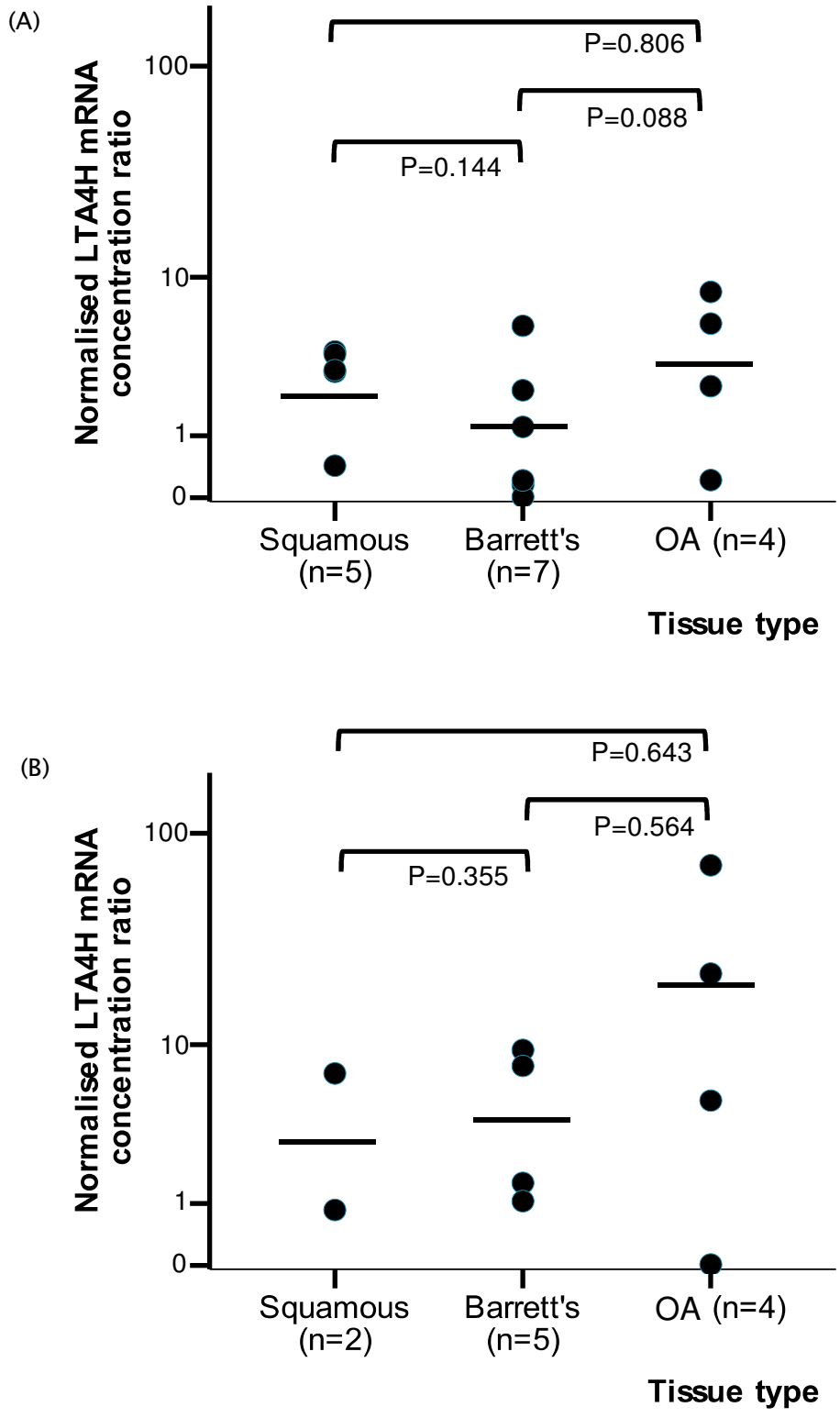


Figure 5-I: *LTA4H* gene transcript levels in (A) Epithelial area (B) Stromal areas.
Gene transcript levels were highest levels in stromal areas, and in particular OA tissue, although this did not reach statistical significance when compared with either squamous control or Barrett's tissue.

5.3.2.4 *LTC4S*

LTC4S gene transcripts were found at recordable levels in only two samples. One sample was from the stromal area of squamous and one from the stromal area of OA tissue. Normalised concentration ratios were 1.92 and 6.15 respectively (figure 5-J). There was no significant difference in *LTC4S* gene transcript levels in epithelial or stromal area between different tissue types.

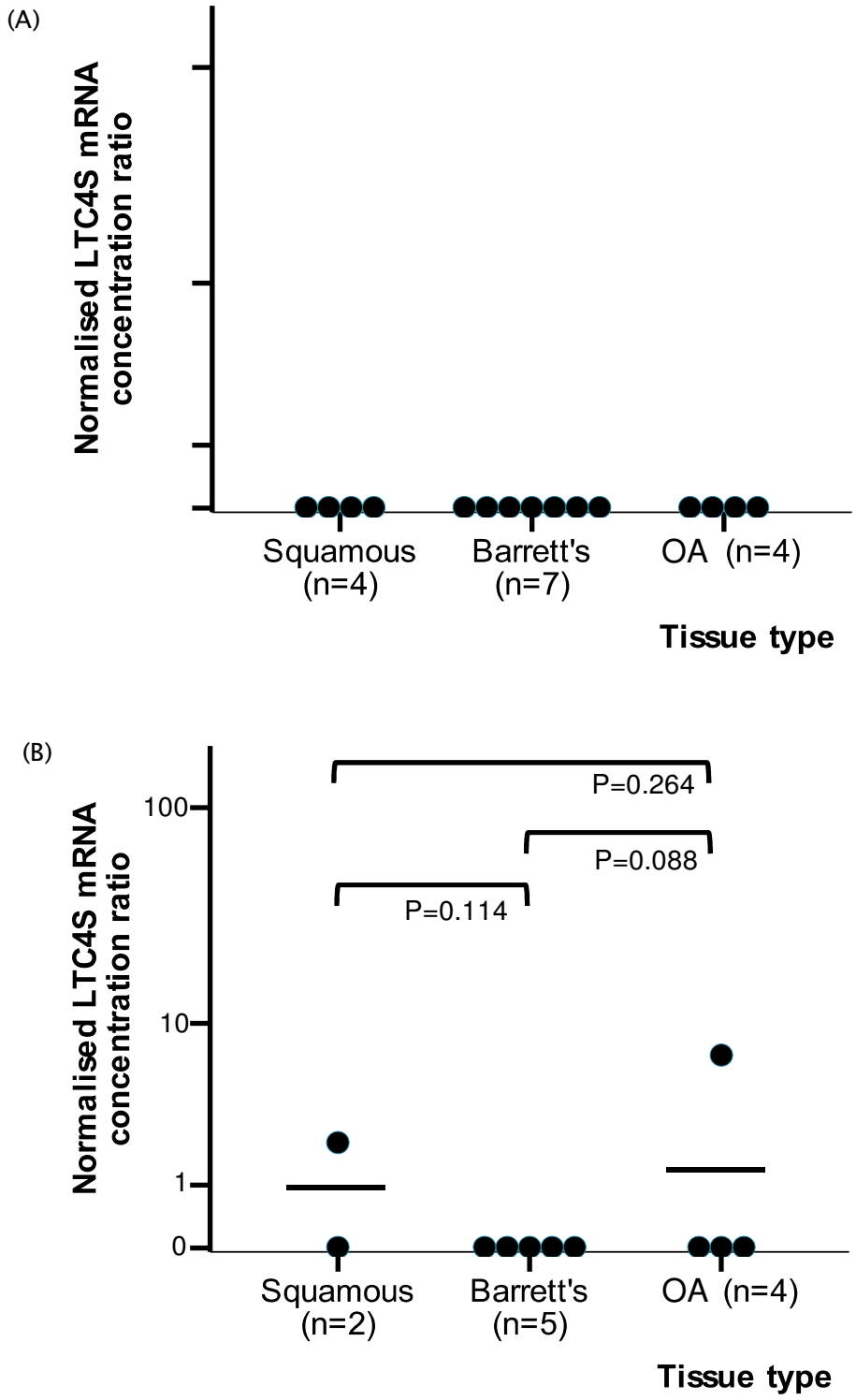


Figure 5-J: *LTC4S* gene transcript levels in (A) Epithelial area (B) Stromal areas
Gene transcript levels were demonstrable in only two samples from the stromal area of tissues; one squamous and one OA tissue.

5.3.2.5 *LTB4R*

LTB4R gene transcript levels were highest in the epithelial region of OA tissue, although this was due to high levels of (37-fold compared to granulocytes) in one sample (figure 5-K). In epithelial areas, mean normalised concentration ratios (mean+/-SD) were 0.12+/-0.15 in squamous, 0.05+/-0.09 in BM, and 9.45+/-18.9 in OA tissue. Transcript levels were low in stromal areas of tissue, with mean normalised concentration ratios of 0.14+/-0.20 in squamous, 0.08+/-0.18 in BM, and zero in OA tissue. Data was distributed non-parametrically and thus the Mann-Whitney U test was used for all statistical comparisons between groups. There was no significant difference in *LTB4R* transcript levels in epithelial or stromal area between different tissue types.

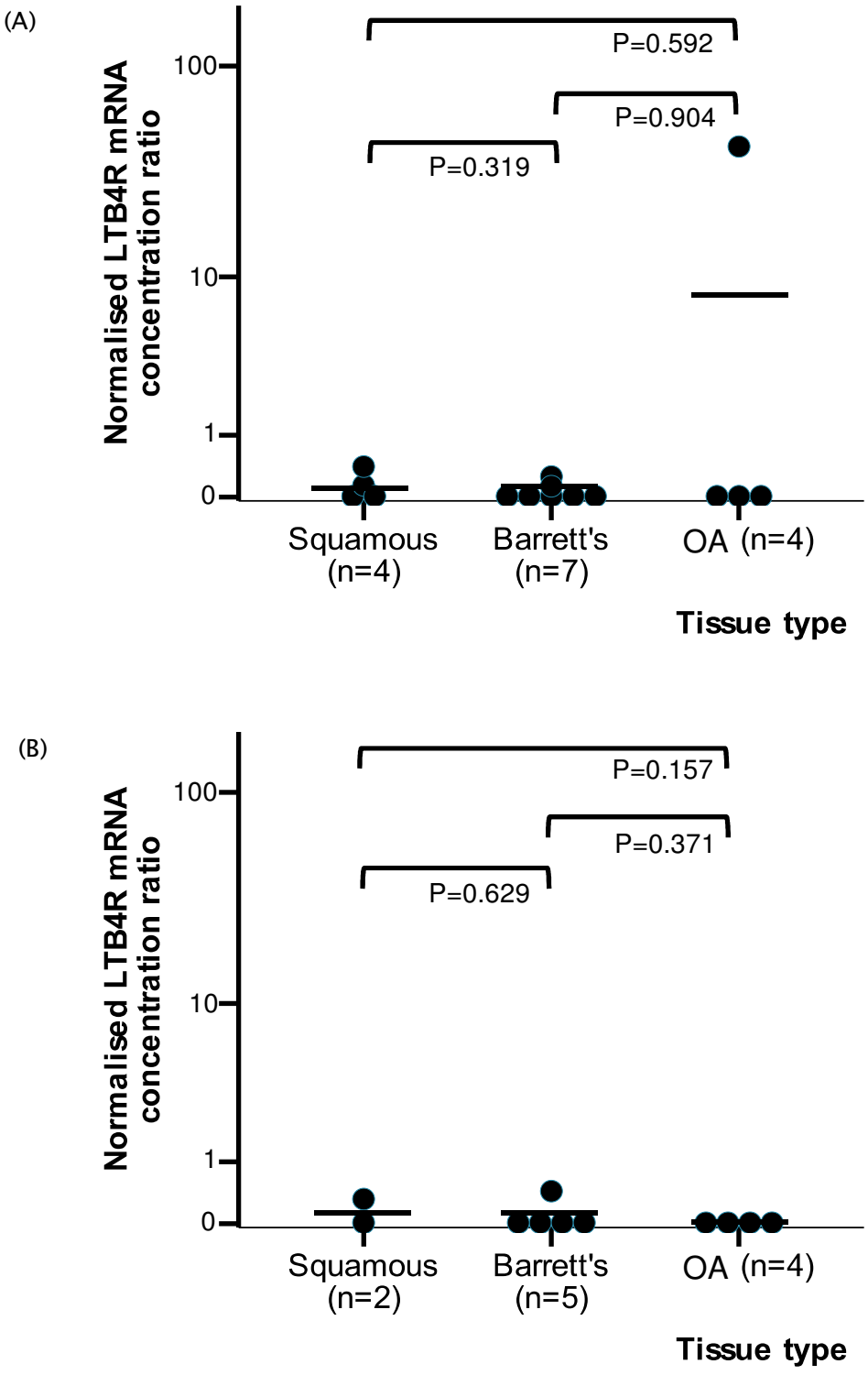


Figure 5-K: *LTB4R* gene transcript levels in (a) Epithelial and (b) Stromal areas.
Gene transcript levels were low in most tissues with the exception of one sample from the epithelial area of OA tissue.

5.3.2.6 *CYSLT1R*

CYSLT1R gene transcripts were found in only small quantities in all tissue types (figure 5-L). In epithelial areas, mean normalised mRNA concentration ratios (mean+/-SD) were 0.16+/-0.13 in squamous, 0.15+/-0.29 in BM, and 0.10+/-0.19 in OA tissue. The highest gene transcript levels were found in stromal area of BM tissue: mean normalised mRNA concentration ratios were 0.07+/-0.09 in squamous, 0.61+/-0.63 in Barrett's, and zero in OA tissue. Data were distributed non-parametrically and thus the Mann-Whitney U test was used for all statistical comparisons between groups. There was no significant difference in *CYSLT1R* gene transcript levels in epithelial or stromal area between different tissue types.

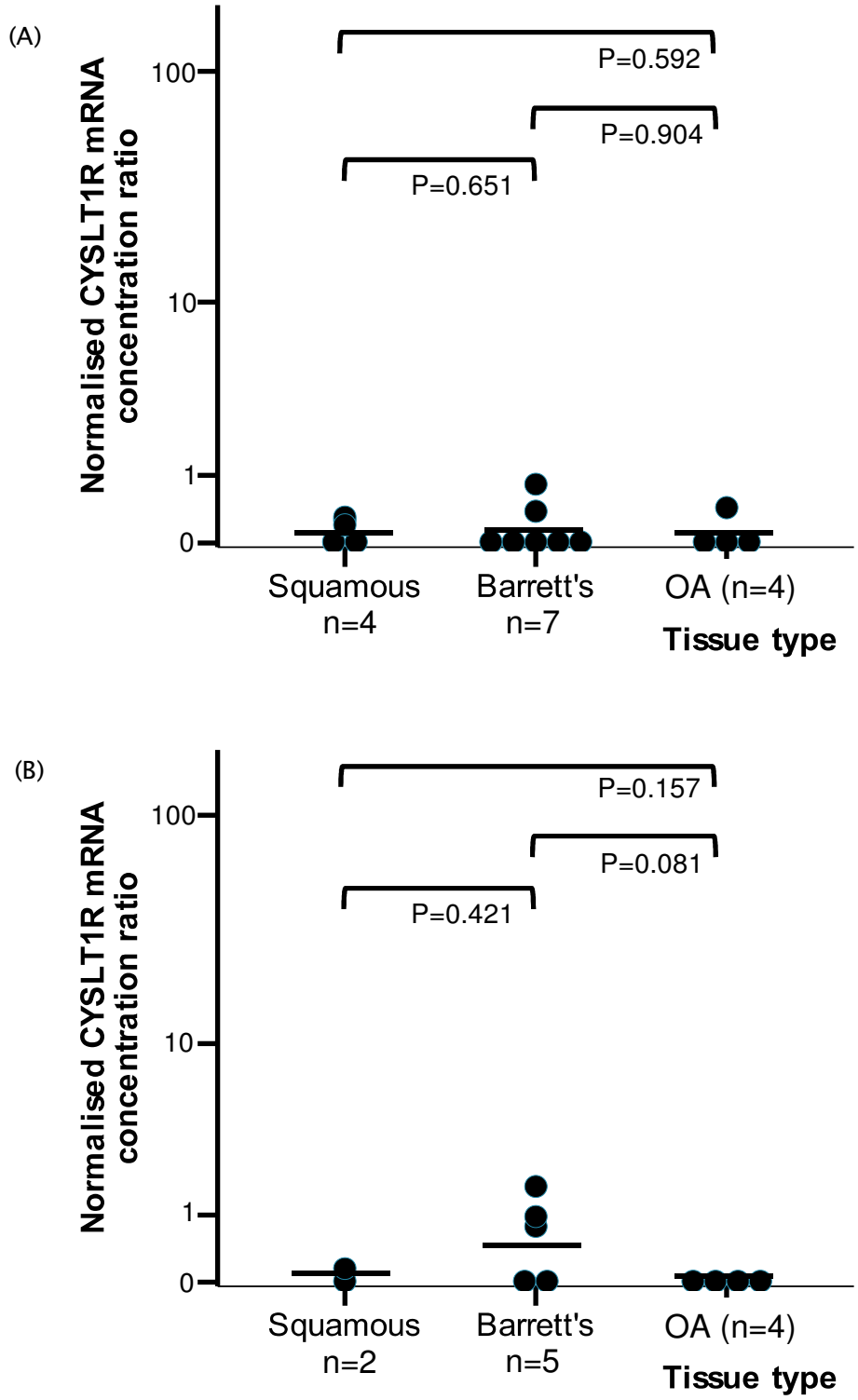


Figure 5-L: *CYSLT1R* gene transcript levels in (A) Epithelial and (B) Stromal areas. Low gene transcript levels were found in both epithelial and stromal tissue areas in all tissue types.

5.3.3 Sample collection and Group demographics

Twenty-eight samples from 16 patients were procured successfully at laser capture micro-dissection, with 7 squamous (5 epithelial, 2 stromal), 12 BM (7 epithelial, 5 stromal), and 9 OA (4 epithelial, 5 stromal). Patient characteristics are shown in table 5-B. There were no significant differences between the groups in age, or aspirin use. No patient in the OA group was taking a PPI at time of endoscopy, compared with 100% usage in both the squamous and BM group.

	Squamous	BM	OA	p value
Mean age in years (median)	64.2 (65.6)	67.5 (68.7)	77.6 (77.5)	ANOVA=0.072
Age Range	51.0-75.8	53.8-77.5	71.9-84.2	-
% male	83.3	85.7	100	X^2=0.098
Aspirin use	0	0	0	-
PPI use	100	100	0	X^2=0.005

Table 5-B: Patient characteristics of laser capture micro-dissected samples.

5.4 Discussion

5.4.1 Protocol validation

Laser capture micro-dissection is an emerging technique for isolating individual populations of cells from tissue to permit downstream mRNA analysis. To produce reliable validated results requires a complex series of steps, each requiring optimisation and validation.

In the first part of this study a robust protocol was formulated and validated for the retrieval of mRNA from frozen biopsy samples of oesophageal tissue for subsequent gene expression analysis. This was successfully achieved, with retrieval of mRNA from tissue samples of squamous, BM and OA. Confidence in the reliability and validity of the formulated protocol can be gained from the multiple normalisation steps that were undertaken. Normalisation is crucial in RT-qPCR to control for experimental error which may be introduced during the multi-stage process required to extract and process mRNA (Huggett et al. 2005). Examples of how this was achieved in this protocol include normalising for the number of cells retrieved during laser capture, ensuring the quantity of mRNA put into the amplification step was the same, and using validated housekeeping genes (see 5.4.1.2). All gene expression assays showed acceptable melt curves (run following real time PCR) signifying that the desired amplicon was detected, and that there was no interference in the results from double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. Efficiencies were also satisfactory across all gene expression assays, with all results between 1.9 and 2.1.

5.4.1.1 Column- was superior to TRIzol® -based method for the isolation of mRNA from laser captured samples

Numerous protocols for the isolation of RNA from laser captured samples exist, and it is recommended each user should optimise and validate their own protocol to ensure reliable results and prevent loss of mRNA (Pinzani et al. 2006). The column based extraction procedure has a number of potential advantages when used with laser captured material. These include the ability to elute RNA in small volumes and to treat with DNase directly onto the column to remove any contaminating DNA without the need for further RNA purification and precipitation. In the TRIzol® based method, RNA is separated from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, and phenol (TRIzol®) and chloroform, followed by centrifugation. Under acidic conditions, RNA remains in the upper aqueous phase, while most of the DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol. Whilst this is a widely accepted and utilised technique, a percentage of the RNA may be lost at

each extraction step and there are a number of theoretical disadvantages when using it on laser captured samples. For example, visualisation of the RNA "pellet" formed during precipitation may be difficult and contribute to RNA loss. In this experiment using the TRIzol® protocol a number of steps were taken to minimise this problem. The aqueous phase was stored with isopropanol at -20°C to improve precipitation of RNA, and glycogen was used to improve visualisation of the RNA pellet. Despite this optimisation the column based method using the RNAqueous-Micro kit (Ambion) yielded a mean of more than 4ng/µl extra total RNA than the TRIzol® based method. This was statistically significant and thus it may be concluded that under the experimental conditions encountered in this study, the column based method is superior.

5.4.1.2 Performance of Housekeeping genes

For interpretation of quantitative gene expression measurements in clinical tumour samples, a normaliser is necessary to correct expression data for differences in cellular input, RNA quality, and reverse transcription efficiency between samples. In many studies, a single housekeeping gene is used for normalisation. However, no unequivocal single reference gene (with proven invariable expression between cells) has yet been identified. In biopsies of human tissue significant variation in expression of commonly used housekeeping genes, including *GAPDH*, *β-actin*,*18s*, and *HPRT1* is found not only between individuals but also between biopsies taken from the same patient (Tricarico et al. 2002). These findings have been corroborated by other studies showing varying gene expression levels in different tissues and under different experimental conditions (Schmid et al. 2003; Radonic et al. 2004). As the best alternative, it is recommended that each housekeeping gene is validated for the individuals' experimental setup and that more than one should be used (Bustin and Nolan 2004). In this study the performance of four pairs of housekeeping genes was assessed for reliability in laser captured samples. The combination of *18s* and *HPRT1* performed least well with a greater than 100-fold difference in gene expression across tissue samples. In contrast, the combination of *18s* and *ACTB* performed with the most reliability in the experimental conditions and with the tissue types in this study, with a less than 10-fold difference. This combination was therefore used in this study, and additionally is recommended for future study.

5.4.2 mRNA transcript levels of 5-LOX pathway enzymes and receptors in Barrett's adenocarcinoma

In general, transcript levels of 5-LOX pathway genes were higher in stromal areas of all tissue types compared with the epithelial area. In particular *ALOX5* was significantly higher in stromal when compared to epithelial areas (p=0.045). When comparing tissue

types, it was in the stromal area of OA tissue that the highest mRNA levels were found. However there was marked variability with some samples showing minimal levels of gene transcripts. For example, only low levels of *ALOX5* transcripts were demonstrated in any tissues. As discussed (see 5.4.1), multiple steps were taken to ensure the experimental protocol was robust, the positive control results rule out failure of real time PCR, and the presence of housekeeping genes in all samples suggest that mRNA degradation is an unlikely cause. Taken together with the immunohistochemistry results (see chapter 4), this would suggest that whilst transcriptional expression of 5-LOX pathway genes is variably increased in OA, other post transcriptional factors appear to play an important role in protein translation. It is recognised that cells use a variety of post transcriptional mechanisms to control protein expression (Bennett et al. 1993). Previous study has demonstrated that mRNA expression may not be predictive of protein expression (Guo et al. 2008). For example, stimulation of human neutrophils with granulocyte macrophage colony stimulating factor upregulated total 5-LOX protein, but did not alter *ALOX5* mRNA (Pouliot et al. 1994).

In contrast to the variability in transcript expression seen with *ALOX5*, *ALOX5AP* and *LTC4S*, *LTA4H* mRNA was found in all tissue types with highest levels in stromal area of OA. LTA_4H is widely and constitutively expressed across tissues and cell types, including neutrophils, monocytes, erythrocytes and epithelial cells in a variety of organs (Haeggstrom 2000). Expression of LTA_4H protein, but not mRNA, has been demonstrated previously in infiltrating neutrophils and epithelial cells in OA (Chen et al. 2003). The study presented here substantiates this, with immunohistochemical studies localising protein expression to neutrophils in the stromal area of OA tissue, with evidence of a non-significant increase in *LTA4H* gene transcription.

The factors responsible for high levels of gene transcripts seen in the stromal areas of OA in some samples are not known. The simplest explanation is that differences in mRNA reflect the variances between tissues in migrating cell populations in which mRNA of the various gene transcripts studied is constitutively expressed. For example, abundant neutrophils were demonstrated in tissues by immunohistochemistry. An alternative explanation may include differences in induction of transcription in the resident population of inflammatory cells. A variety of inducers of transcription of 5-LOX pathway genes are known, including retinoic acid, 1,25-dihydroxyvitamin D_3 (calcitriol), colony stimulating factors, transforming growth factor- β (TGF-β), IL-1 and IL-4, although their ability to upregulate transcription varies between tissue types (Peters-Golden and Brock 2003). TGF-β is of particular interest as it plays a central role in cancer pathogenesis, suppressing the proliferation of normal epithelial cells in early carcinogenesis, while promoting the acquisition of invasive properties by already

transformed cells (Weinberg 2007). In OA, overexpression of TGF-β is linked to an advanced stage of disease and a poor prognosis (Koliopanos et al. 2002; von Rahden et al. 2006), whilst impairment of TGF-β mediated growth suppression signalling through inactivation of SMAD4, has been demonstrated in both BM and OA (Onwuegbusi et al. 2006). TGF-β can modify maturation of *ALOX5* transcripts leading to augmented expression of the functional enzyme, and thus may provide the link by which 5-LOX pathway protein expression is upregulated in OA tissue (Harle et al. 1998). This study did not categorise patients into survival categories, and it is possible that variations in TGF-β levels may play a role in the variations of 5-LOX pathway enzymes gene transcript levels seen in this study.

Further explanation may be the presence of genetic polymorphisms, which may contribute to increased transcription found in some samples. For example, eosinophils from subjects with a single genetic polymorphism in the *LTC4S* gene produced three times more LTC_4S than subjects with the wild-type genotype (Sampson et al. 2000). How common such polymorphisms are in patients with BM or OA is not known, but in certain other disease subgroups, such as aspirin intolerant asthma, the prevalence may be as high as 76% (Sanak et al. 1997). In colorectal cancer, no association was found between two *5-LOX* polymorphisms and risk of colorectal cancer, but a protective effect was demonstrated between a polymorphism in *12-LOX* (Gong et al. 2007). Conversely, in patients with breast cancer, *ALOX5AP* -4900 A>G polymorphism and high dietary linoleic acid intake was linked with an increased risk of breast cancer (Wang et al. 2008). This inconsistency between tumour types may be explained by promoter polymorphisms. For example in the *ALOX5* promoter which may account for a lack of 5-LOX enzyme in approximately 5-6% of the population who possess homozygous recessive genotypes (Drazen et al. 1999). Further study is needed and exploring the frequency of such polymorphisms in patients with BM and OA may provide an insight into the causation of this disease in some and not others.

5.4.3 mRNA transcript levels of the leukotriene receptors: *LTB4R* and *CYSLT1R*

The finding of high transcript levels of *LTB4R* mRNA in cells in the epithelial area of OA tissue was unexpected. Confidence that this result is not due to infiltrating white cells can be gained by the low levels of *PTPRC* transcripts seen in the epithelial samples. In non-disease states BLT1 receptors are expressed on leucocytes, and on activation by LTB_4 play a key role in leukocyte recruitment to sites of inflammation by both directing cellular migration, and by facilitation of endothelial cell binding, a prerequisite of leukocyte migration into tissues (Tager and Luster 2003). A clue to its potential role in carcinogenesis comes from the finding that LTB_4 prolongs neutrophil survival by preventing apoptosis (Hebert et al. 1996). An increasing role for BLT1 receptors has

been found in multiple cancer types. In pancreatic cancer tissue, increased levels of *LTB4R* mRNA were found when compared with normal pancreatic tissue (Hennig et al. 2002). Subsequent findings demonstrated that LTB_4 can stimulate proliferation and activation of the signalling molecules, extracellular signal-regulated kinase (ERK) 1/2 in multiple human pancreatic cell lines (Tong et al. 2002). In human anaplastic large cell lymphoma cells the LTB_4 receptor antagonist, LY293111 (etalocib), induces cell cycle arrest and apoptosis via c-Jun N-terminal kinase (JNK) phosphorylation (Zhang et al. 2005). In the human gastrointestinal tract, BLT1 receptor was found to be expressed in colon cancer cells and blockade by the specific BLT1 receptor antagonist U75302 induced apoptosis via inhibition of ERK activation (Ihara et al. 2007). In oesophageal cancer cell lines the 5-LOX inhibitor AA861 suppressed LTB_4 production and the BLT1 receptor antagonist U-75302, blocked AA861-induced apoptosis (Hoque et al. 2005). This finding was variable across the small number of tissues investigated and thus further work is required to clarify this result, which should include corroboration with immunohistochemical analysis.

Transcripts for *CYSLT1R* were seen in the stromal area of some BM tissue samples, with no expression seen in the stromal area of OA tissue. CysLT1R expression has been documented in other sites of the gastrointestinal tract; the small intestines and in the colon, in colorectal carcinoma (Lynch et al. 1999; Ohd et al. 2003). In colorectal cancer the expression was predominantly in the epithelial cells of tumour tissue compared with controls taken from the margins of the surgical specimens (Ohd et al. 2003). In the study in this thesis the explanation for expression of *CYSLT1R* is likely the presence of white cells, and in particular monocytes/macrophages, mast cells and eosinophils in the stromal area, all known to express cysLT1 receptors. Stimulators of transcription of *CYSLT1R* in monocytes and macrophages include interleukin-4 (Thivierge et al. 2001). Four fold increase in the level of this cytokine was found in BM when compared to both normal squamous and oesophagitis tissue, which may provide an explanation for the higher levels of expression demonstrated in BM tissue (Fitzgerald et al. 2002b). Its functional role is not demonstrated in this study, but cys-LTs are known to have diverse roles in the inflammatory response acting through the cysLT1 receptor (Kanaoka and Boyce 2004). In BM it may contribute to mediation of leukocyte activation and chemotaxis thus contributing to the inflammatory infiltrate demonstrated by immunohistochemistry. A further role may be promotion of cell proliferation through downstream phosphorylation of ERKs, contributing to an environment conducive to cell growth (Mellor et al. 2002).

5.4.4 Conclusions

In this study a reliable and validated protocol was developed for the procurement of individual cellular areas from both diseased and non-diseased oesophageal tissue by laser capture microdissection. Utilising this protocol for a pilot assessment of 5-LOX pathway gene expression in squamous, BM and OA tissue has confirmed mRNA transcripts for all four 5-LOX pathway genes, most consistently (but not exclusively) in stromal areas of OA tissues, with *LTA4H* mRNA also being prominent in epithelial cells. Additionally, mRNA expression of the two main leukotriene receptors, *LTB4R* and *CYSLT1R* were demonstrated in epithelial and stromal areas respectively of some samples. Both receptors may mediate leucocyte recruitment and play a role in promotion of cell growth. Results were variable, and taken with the findings from the immunohistochemical studies, suggest that post transcriptional regulation of protein translation is important.

CHAPTER 6: Cost-utility analysis of radiofrequency ablation or oesophagectomy for the management of high-grade dysplasia in Barrett's metaplasia

In this chapter the results of the cost-utility analysis are presented and discussed with a summary found in section 6.1. Results of the base case analysis are in section 6.2, deterministic analysis 6.3, and probabilistic analysis 6.4. These results are discussed in section 6.5.

6.1 Summary

In the base case analysis radiofrequency ablation (RFA) dominated as it generated 0.4 extra quality of life years (QALYs) at a cost saving of £1902. For oesophagectomy to be the most cost-effective option would require a RFA treatment failure rate (high-grade dysplasia persistence or progression to cancer) of greater than 44%, or an annual risk of high-grade dysplasia (HGD) recurrence or progression to cancer in the ablated oesophagus of greater than 15% per annum. There was an 85% probability that RFA remained cost-effective at the NICE willingness to pay threshold range of £20000-30000.

RFA is likely to be a cost-effective option for HGD in Barrett's metaplasia (BM) in the UK. Long term outcome data are required to achieve certainty as to the optimal management strategy, as current follow up data is limited to two years. However, the model presented here predicts that if initial eradication of high-grade dysplasia is achievable in over 60% of patients, then RFA with oesophagectomy for HGD persistence or recurrence will be the preferred strategy.

6.2 Deterministic Model - Base case analysis

6.2.1 Summary

The results from the base case analysis are summarised in table 6-A. For a 64 year old male patient, a strategy of RFA followed by oesophagectomy if there was disease recurrence would be expected to cost £1902 less, and result in 0.4 more QALYs, when compared with immediate oesophagectomy.

Outcome per strategy	Oesophagectomy	RFA	Incremental ratio
Cost (£)	8555	6653	-1902
Unadjusted life-years	17.0	17.4	
QALY	13.8	14.2	0.4
Incremental cost-effectiveness ratio (£/QALY)		Dominates	

Table 6-A: base case results of the deterministic model.
RFA was cheaper and resulted in more QALYs than oesophagectomy.

Figure 6-A shows the cost-effectiveness plane which plots incremental effectiveness against incremental cost. The base case result falls in the southeast quadrant, and thus the RFA strategy is said to dominate immediate oesophagectomy.

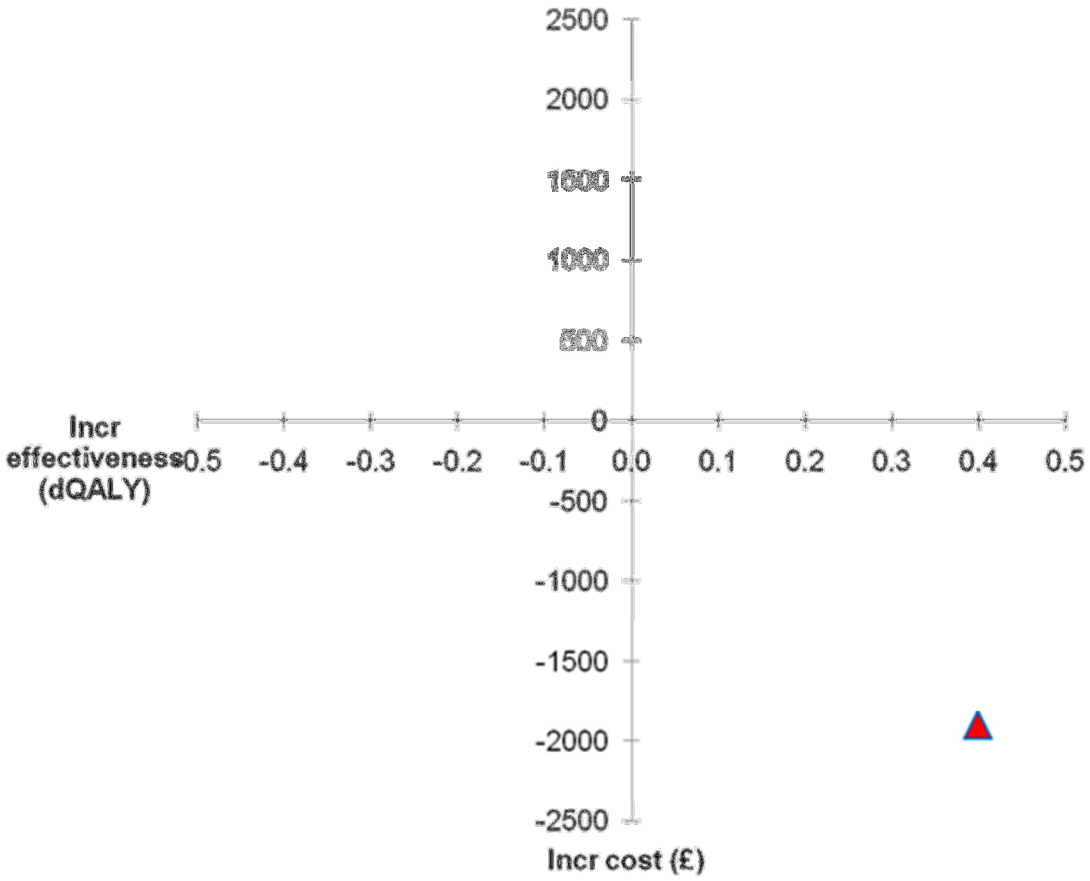


Figure 6-A: Base case analysis – cost-effectiveness plane.

The result lies in the south east quadrant of the cost-effectiveness plane and thus is said to dominate oesophagectomy.

6.2.2 Morbidity

The RFA strategy results in less morbidity (20.8% v 87.1%) than immediate oesophagectomy, as shown in table 6-B.

	Oesophagectomy strategy (% patients)	RFA strategy (% patients)
Procedural	37.1	3.9
Strictures	50.0	16.9

Table 6-B: Base case analysis - morbidity

6.2.3 Mortality

The RFA strategy resulted in less procedure related mortality (0.4% v 2.6%) than immediate oesophagectomy. The causes of death as a percentage of total deaths are shown in table 6-C.

	Oesophagectomy strategy (% deaths)	RFA strategy (% deaths)
Age-related	96.43	99.09
Surgical	1.89	0.19
Endoscopic	0.71	0.18
Cancer	0.97	0.53

Table 6-C: Base case analysis - cause of death

10.18% of the RFA cohort required an oesophagectomy for recurrence of HGD or progression to OA and of those, 3.93% died of terminal cancer, compared to 0.73% of those in the immediate oesophagectomy strategy. Age-related deaths were by far the most common cause of mortality in both cohorts, with cancer the cause of death in less than 1% of patients. Table 6-D shows 5-year survival and cancer free survival by cohort, and figure 6-B shows the survival curve (otherwise known in the context of health economic modelling as a Markov trace) of the base case analysis with population norms included for illustrative purpose. Both strategies appear to be highly effective at preventing death from cancer as demonstrated by the similarity between 5-year survival and 5-year cancer free survival figures.

	Oesophagectomy	RFA
5-year survival	84.89	84.99
5-year cancer free survival	85.03	85.07

Table 6-D: Base case analysis - 5-year survival

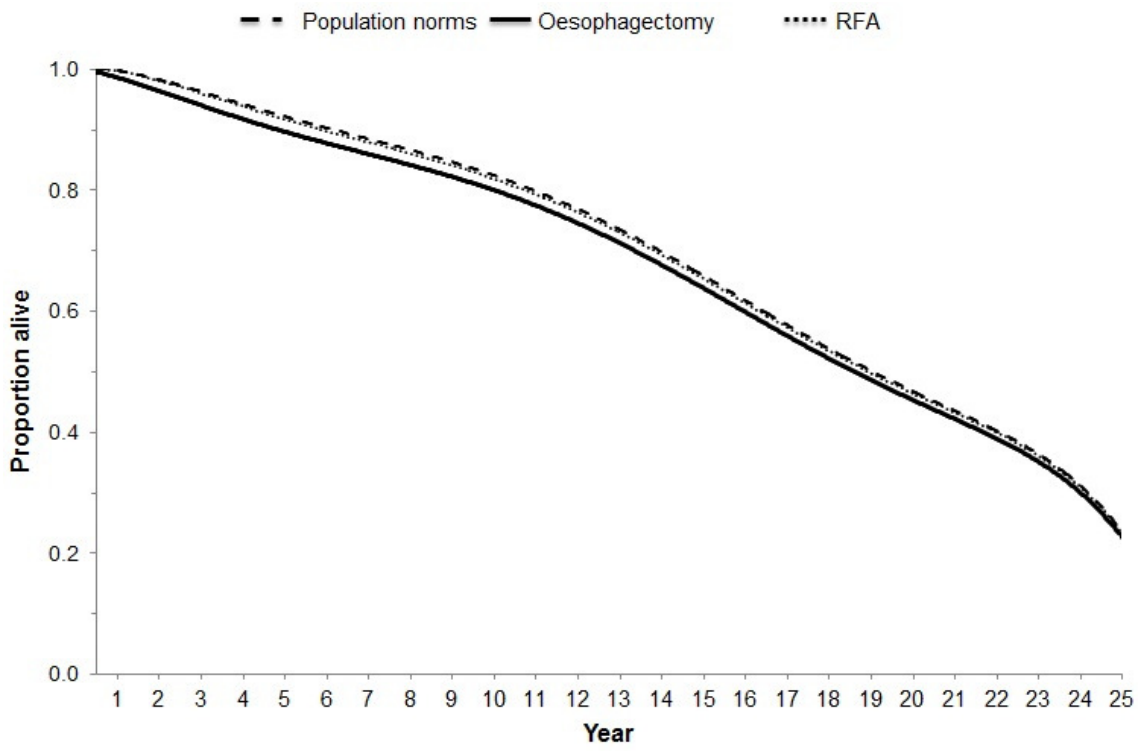


Figure 6-B: Base case analysis - survival curve (Markov trace)

6.3 Deterministic sensitivity analysis

6.3.1 Summary

The one-way sensitivity analysis demonstrated that the following were important factors in determining the cost-effectiveness of RFA; outcome post RFA, utility post RFA with squamous oesophagus, and utility fully recovered post oesophagectomy.

6.3.2 Outcome post RFA

Only at the most pessimistic outcome post RFA (i.e. 30% post RFA with HGD), was RFA more costly than oesophagectomy, although it still resulted in more QALYs, costing £661/QALY. Threshold analysis demonstrated that for oesophagectomy to dominate as the preferred strategy required over 44% of patients to have residual HGD post RFA. This is demonstrated in the cost-effectiveness plane (figure 6-C), which shows the incremental cost and incremental QALY as the transition probability for outcome after RFA is HGD is altered throughout a hypothetical range.

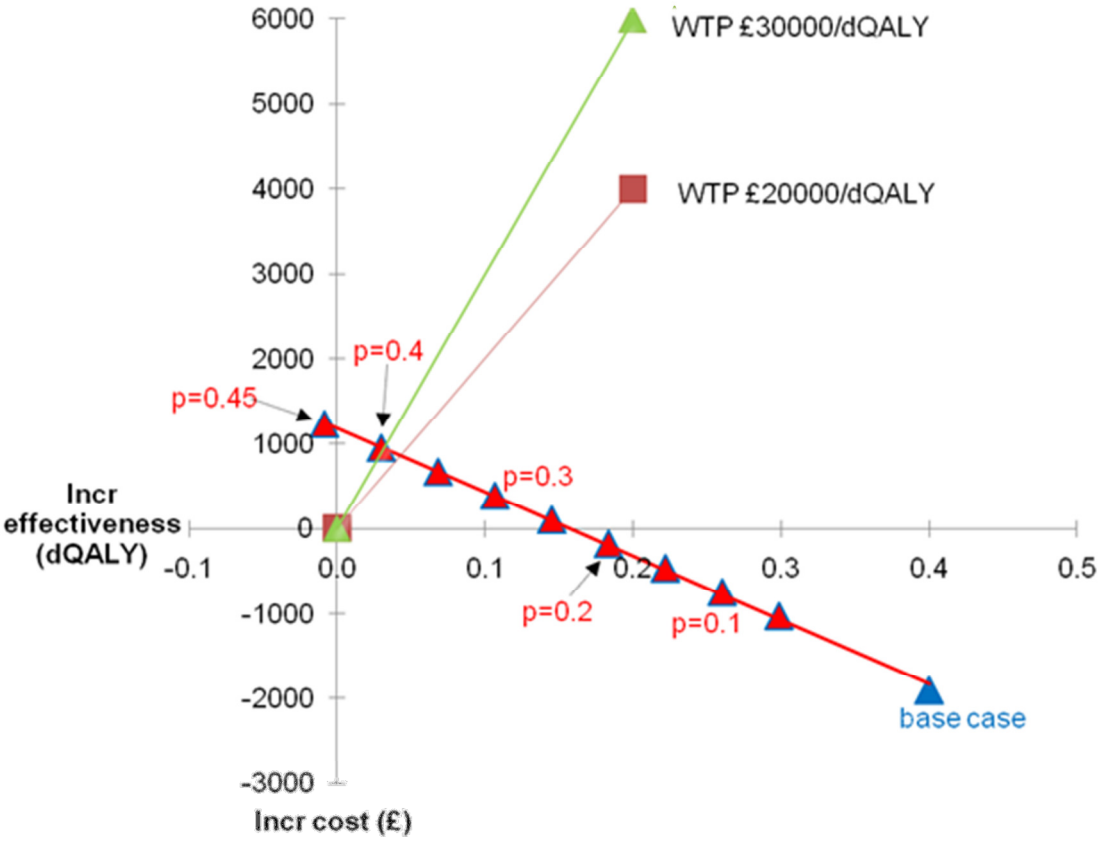


Figure 6-C: Threshold analysis – altering transition probability of outcome after RFA is HGD

Abbreviations: WTP – willingness to pay, dQALY – discounted quality of life years

6.3.3 Utility

Utility values were altered as highlighted by the one-way sensitivity analysis. For oesophagectomy to become a more effective (but still more costly) option would require either a utility value of less than 0.93 post RFA with squamous oesophagus, or a utility fully recovered post oesophagectomy of over 0.99. Thus, it would require fully recovered post oesophagectomy to be a more preferable health state than post RFA with squamous oesophagus.

6.3.4 Recurrence of HGD or progression to oesophageal adenocarcinoma after successful RFA

As there is uncertainty in the robustness of the neo-squamous epithelium post RFA, further threshold analysis was undertaken to identify the HGD recurrence required to alter the results of the model. For this to occur would require an annual risk of HGD recurrence in the neo-squamous epithelium of 20%. This is demonstrated in the cost-effectiveness plane (figure 6-D), which shows the incremental cost and incremental

QALY as the transition probability for recurrence of HGD or progression to OA during post RFA surveillance is altered throughout a hypothetical range.

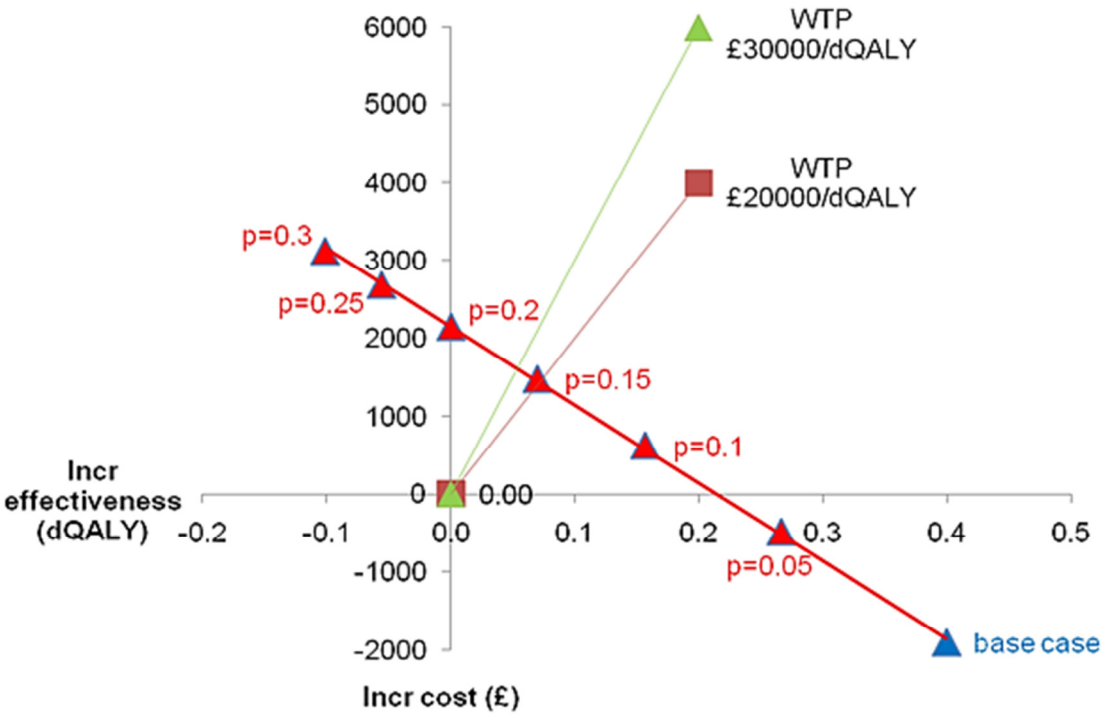


Figure 6-D: Threshold analysis – altering transition probability of recurrence of HGD or progression to OA during post RFA surveillance
Abbreviations: WTP – willingness to pay, dQALY – discounted quality of life years

6.3.5 Endoscopic surveillance

The need for, or frequency of, endoscopic surveillance after successful ablation with RFA is not currently known. A scenario was modelled of continuing surveillance in all patients post RFA, with 2-yearly endoscopy for both neo-squamous and BM, after initial yearly endoscopy for the first 5 years.

With this strategy the cost of RFA increased considerably to £8168 with 14.2 QALYs. The cost of oesophagectomy remained at £8555 with 13.8 QALYs, and thus RFA still dominated as the preferred strategy.

6.3.6 Costs

As the method used to calculate the cost of RFA differed from other calculated costs, a threshold analysis was undertaken to determine how expensive treatment with RFA would need to be to alter the outcome of the model. For RFA to become a more expensive treatment strategy would require an increase of £1904 from the base case estimate to £5773 per treatment course per patient. RFA retained its cost-effectiveness at a willingness to pay (WTP) of £30000/QALY even if cost was increased to £17754,

i.e. 450% more than the base estimate and over twice the cost of an oesophagectomy without complications. Altering all other variables through their specified range, including age (55-70yrs), and in addition altering the discount rate (1-5%), and the length of time for which the cohort was followed (5-30yrs), made no critical difference to the model outcome.

6.4 Probabilistic sensitivity analysis

6.4.1 Base case results

The base case results of the probabilistic sensitivity analysis (see table 6-E) are similar to the deterministic model. The RFA strategy is seen to dominate as it costs less and provides more dQALY than immediate oesophagectomy.

	Cost	dQALY
Oesophagectomy	£8,546	13.85
RFA	£6,661	14.24
Incremental	-£2,048	0.32

Table 6-E: Probabilistic sensitivity analysis – Base case results

6.4.2 Cost-effectiveness plane

The cost-effectiveness plane demonstrating the distribution of the results is shown in figure 6-E. This scatter plot of the incremental costs and QALY pairs, generated from the probabilistic sensitivity analysis, illustrates the uncertainty surrounding the estimates of expected costs and expected effects associated with the RFA compared with the Oesophagectomy strategy. The majority fall in the south-east quadrant, the position where the RFA strategy is more effective and less costly than the oesophagectomy strategy, and thus RFA is said to dominate.

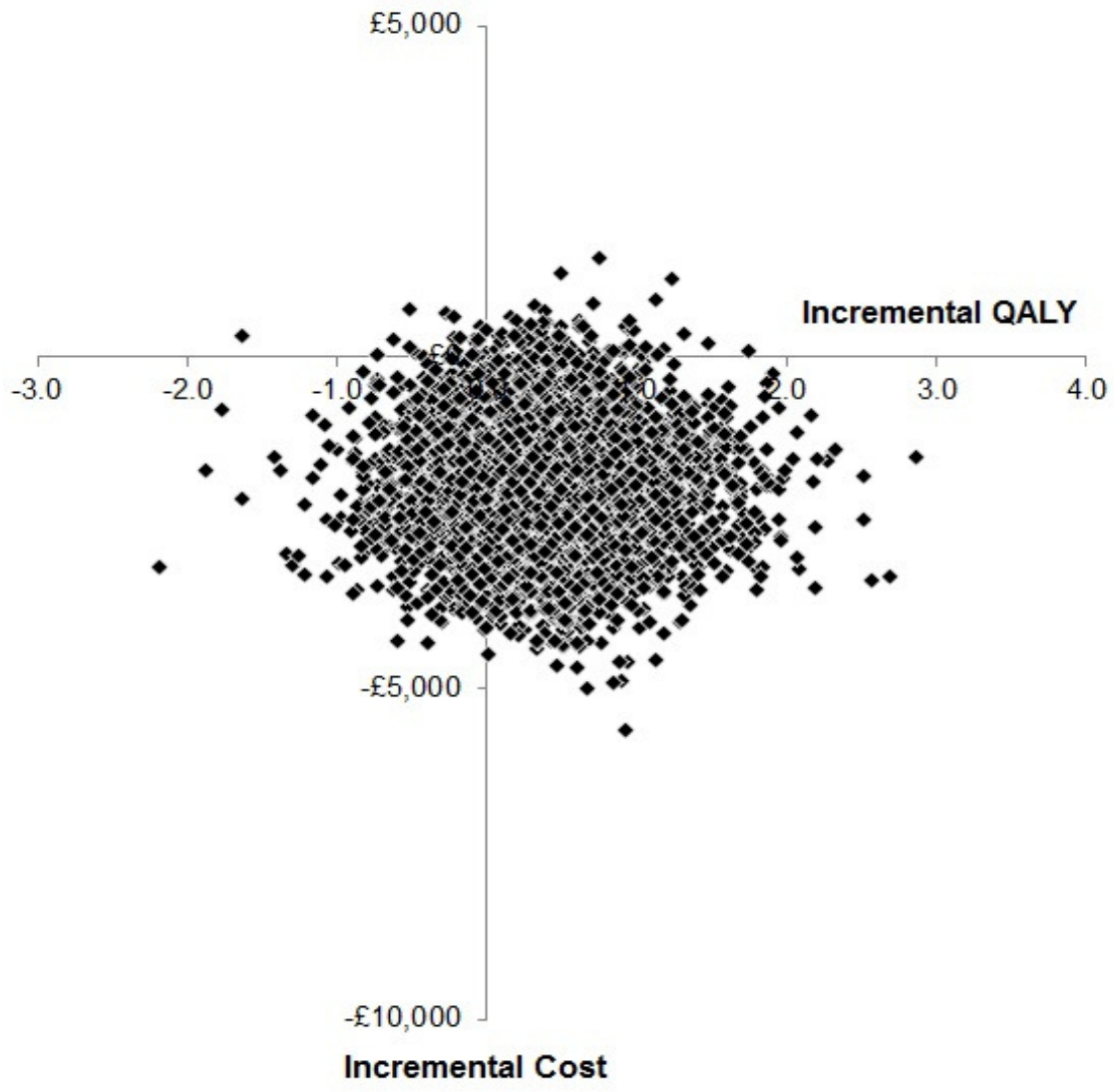


Figure 6-E: Cost-effectiveness plane of results of probabilistic sensitivity analysis

6.4.3 Cost-effectiveness acceptability curve

The cost-effectiveness acceptability curve shows the probability that RFA is cost-effective as the value placed upon a QALY is varied between £0 and £50,000 (figure 6-F). The probability that RFA is cost-effective is approximately 84-86% throughout a willingness to pay of £20000-30000/QALY, showing that RFA appears highly likely to be cost-effective.

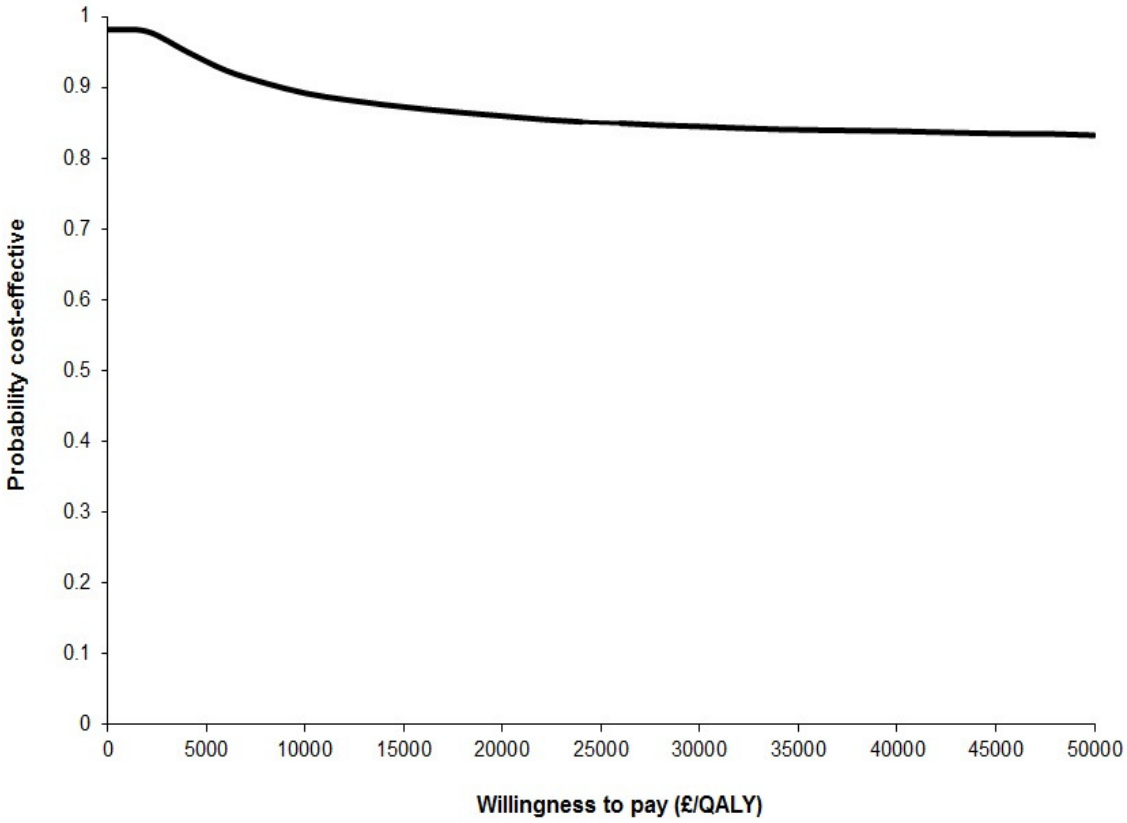


Figure 6-F: Cost-effectiveness acceptability curve

6.5 Discussion

6.5.1 Summary

This study demonstrates that for a 64 year old patient in the UK with HGD in BM, a treatment strategy of RFA followed by surveillance with oesophagectomy for HGD recurrence or progression to adenocarcinoma is less expensive and results in more discounted quality adjusted life years compared to immediate oesophagectomy. This supports findings which demonstrated that in a US setting, a strategy of RFA for HGD yielded more QALYs at less cost than surveillance, oesophagectomy, or other ablative strategies, with the exception of photodynamic therapy (PDT), which gave comparable QALYs but at a higher cost (Inadomi et al. 2009).

6.5.2 Identification of areas of uncertainty

One of the roles of a health economic analysis is to identify areas of uncertainty which may guide future study. Key areas have been identified by this study. The first concerns the success rate achievable by RFA in eliminating HGD from BM. Data thus far show that RFA is very effective at eliminating HGD with success in 90% or more of cases, and with removal of BM in between 60-91%. The modelling demonstrated that RFA remains cost-effective even if eradication of HGD is achievable in only 60% of cases at a willingness to pay of £30000/QALY, a figure well below the 90-97% in published trials (Ganz et al. 2008; Gondrie et al. 2008a; Pouw et al. 2008; Sharma et al. 2009; Shaheen et al. 2011).

Secondly, the one-way sensitivity analysis suggested that utility of patients post RFA with neo-squamous oesophagus would need to be lower than utility of those post oesophagectomy. Utility values for patients undergoing RFA have not yet been derived, although there is some preliminary evidence that patients with resolution of dysplasia post RFA have an improvement in surrogate markers of quality of life, such as depression, stress, and difficulty sleeping as measured using a visual analogue scale (Shaheen et al. 2008). Conservative estimates of utility were used in the study in this thesis, based around known values ascertained by standard gamble technique, considered a robust method for deriving utility values (Drummond et al. 2005). Although minimally invasive techniques for oesophagectomy are improving, resulting in lower morbidity and mortality, it is unlikely that a surgical approach would be a favoured patient preference (Gemmill and McCulloch 2007).

6.5.3 Comparison with health economic evaluation on ablative therapies by NICE

A recent health economic evaluation by NICE looking at ablative therapies for treatment of HGD in BM has demonstrated that RFA with subsequent surveillance resulted in more QALYs but was considerably more expensive than oesophagectomy

(NICE Aug 2010). This in part contrasts the findings presented here, where although the cost of RFA increased considerably with surveillance, it was still cheaper than oesophagectomy with more QALYs, and thus still dominated oesophagectomy as the preferred strategy. The explanation for the lack of parity was the inclusion of the cost of proton pump inhibitors in their model in the post RFA, but not in the post oesophagectomy strategy. This would unfairly bias the study in favour of oesophagectomy for two reasons. Firstly, published quality of life data have suggested that up to 75% of patients post oesophagectomy still complain of reflux symptoms, and this would likely have a major impact on cost incurred for medication usage, and potentially on visits to their general practitioner or hospital consultations (Lagergren et al. 2007). Secondly, in their model no allowance has been made for poor compliance, which would lead to falsely high costs in the RFA strategy.

6.5.4 Endoscopic surveillance after ablation with RFA

The need for or frequency of endoscopic surveillance after successful ablation with RFA for HGD is not currently known. The long term stability of the neo-squamous epithelium after RFA for HGD is not yet determined and this is likely to lead to clinicians continuing surveillance on their patients, despite apparent histological resolution. In the model presented here, although the cost of RFA strategy increased considerably, it still dominated the oesophagectomy strategy. In both the NICE health economic model and the published US study, surveillance considerably added to the cost of ablative therapies, and made RFA a more expensive strategy than oesophagectomy (Inadomi et al. 2009; NICE 2010b). The frequency and duration of follow up endoscopy for patients who have undergone successful ablation will thus need to be considered very carefully, once the durability of the neo-squamous mucosa has been determined with long term follow up data.

6.5.5 Other ablative therapies

Other management strategies for treating HGD in BM exist such as surveillance alone, PDT or other ablative therapies. In the UK surveillance of those with HGD is not presently practiced. Several previous cost-effective analyses comparing ablative therapy to surveillance have found surveillance to be costly and inefficient at preventing progression to cancer (Shaheen et al. 2004; Vij et al. 2004; Inadomi et al. 2009). Studies from the US have demonstrated PDT is an effective alternative to oesophagectomy, but at a cost varying between $6000 and $50000/dQALY. However, acceptance of PDT as an option in the UK has been hampered by a stricture rate as high as 35% in some series, and the ultimate progression to cancer in 15% (Overholt 2007). Comparatively, the acceptance of RFA as a realistic option is substantiated by its low complication rate, demonstrated further by the model presented here, in which

the procedure related morbidity was 6%, compared with 50% for oesophagectomy. In addition, given the data available it appears effective at preventing cancer deaths, with terminal cancer the cause of death in only 0.5% of the hypothetical cohort. Currently no patient in reported trials has progressed to inoperable cancer, but longer periods of follow up are needed before this apparently low risk can be confirmed.

6.5.6 Model assumptions

6.5.6.1 Barrett's oesophagus disease states

There is an established progression from BM through low grade dysplasia (LGD) and HGD to invasive adenocarcinoma. For the purpose of this model BM and LGD were grouped together. This assumption was based on recent data which has suggested that LGD did not appear to confer an increased risk of developing cancer (Sharma et al. 2006). In fact the majority regressed to BM or went back and forth between LGD and BM. In addition, the histological classification and inter-observer agreement in the diagnosis of LGD is variable, with factors such as inflammation playing an important role in misclassification.

Another assumption is not distinguishing between uni- and multifocal HGD, in accordance with the other economic analyses in this area (Inadomi et al. 2009; NICE Aug 2010b). There is evidence to suggest that patients with multifocal HGD are more likely to have a high risk of progression to adenocarcinoma compared with patients with unifocal HGD (Buttar et al. 2001). Even with this increased risk it is felt unlikely this would affect the outcome of this model, given the outcome of the threshold analysis. In addition these findings were not supported by another retrospective study (Dar et al. 2003). The parameters used in the model in this thesis included patients with both histological subtypes, and thus one would expect allowance for the potential increase risk with multifocal HGD.

6.5.6.2 Exclusion of intra-mucosal cancer

Those with T1 disease were excluded from this study. Endoscopic resection of OA confined to the mucosa (T1a) is practiced in the UK, given the low likelihood of lymph node invasion (Stein et al. 2005). However, there is a high risk of recurrent disease, with cancer-free survival of 80% in one series (although these patients were able to have retreatment endoscopically without influencing survival), compared to 97% of those undergoing an immediate oesophagectomy (Prasad et al. 2009). There are preliminary data suggesting RFA of the remaining Barrett's segment after endoscopic resection of the T1a lesion can be achieved with favourable outcomes (Pouw et al. 2008). Follow up was limited to a median of 21 months, during which one patient (of 16 in the cohort with OA) developed disease infiltrating into the submucosa (stage

T1b), which may be associated with lymphatic spread in 20% (Stein et al. 2005). The quality of life, costs, and outcomes involved in such a treatment strategy are complex, and the health economic argument is therefore best served by a separate analysis.

6.5.7 Conclusions

Randomised controlled trials are generally considered the most robust method of determining the benefit of a new treatment (Sibbald and Roland 1998), and certainly one could conclude that a randomised control trial of oesophagectomy versus RFA would be the next logical step. However randomised control trials are costly and time consuming. The results of the model support the case that RFA should be the optimum strategy and only with pessimistic parameters does this outcome change. The model may be influenced by the outcome after RFA, and thus prospective long-term outcome data from a well-constructed clinical trial, with population matched controls, such as the on-going AIM-dysplasia sham-controlled trial in the US, should be sufficient to confirm that RFA is the most (cost-) effective strategy. The critical issue then is whether clinicians would still have equipoise over the two strategies despite the findings of the modelling; if so a definitive trial would still be needed.

In conclusion, the study presented here has suggested that a strategy of RFA with oesophagectomy for HGD recurrence is a cost-effective strategy when compared with immediate oesophagectomy for the management of BM with HGD in the UK. Sensitivity analysis demonstrates that RFA is likely to remain the preferred option, providing that removal of HGD in BM is achievable in over 60%. Long term prospective outcome data will help clarify whether RFA should become the first line treatment option for HGD in BM in the UK.

CHAPTER 7: Conclusions and future study

The aim of this thesis was two-fold, firstly to facilitate greater understanding of the cellular sites of 5-lipoxygenase (5-LOX) pathway expression in Barrett's metaplasia (BM) and oesophageal adenocarcinoma (OA), and secondly to identify whether radiofrequency ablation (RFA) of high-grade dysplasia in BM, compared to oesophagectomy, is a cost-effective option in the UK. Both of these aims were achieved, and shed light onto these two important areas, as well as provoking questions for further study.

7.1 Expression of The 5-LOX pathway in Barrett's metaplasia and oesophageal carcinogenesis and impact on future research

There are numerous examples of chronic inflammation in an organ increasing the risk of future malignant change. Chronic inflammation in Barrett's oesophagus, particularly with regards to cyclo-oxygenase pathway, has been well studied. The alternative leukotriene pathway has not received similar attention. Since Feldberg and Kellaway (1938) introduced the name "slow reacting substance (of anaphylaxis)" for a smooth muscle-contracting factor in the perforate of guinea pig lung treated with cobra venom, diverse roles have been identified for the 5-LOX pathway and its lipid products, the leukotrienes. In particular, evidence is building for an important role in the development of numerous tumour types in humans. The studies detailed in this thesis have added to this evidence and suggest a role for the 5-LOX pathway in BM and OA. These results and studies in other tissues provoke interesting hypotheses and a proposed role is illustrated in figure 7-A.

The role of infiltrating inflammatory cells is important in carcinogenesis with the production of inflammatory mediators playing a significant role. The studies in this thesis suggest a particular role for neutrophils in the stromal area of OA tissue where they express 5-LOX pathway proteins involved in the biosynthesis of LTB_4 from free arachidonic acid. They suggest also the synthesis of LTB_4 and cys-LTs by LTA_4 hydrolase and LTC_4 synthase within OA epithelium.

The molecular factors initiating the upregulation of 5-LOX pathway protein expression in BM and OA warrant further study. Transforming growth factor β (TGF-β) and other cytokines and growth factors may play a significant role (Harle et al. 1998). Both

macrophages and mast cells are resident within oesophageal tissue and possess the capacity to respond to exogenous triggers and generate both LTB_4 and cys-LTs, alongside other mediators, that orchestrate the subsequent inflammatory response and its subsequent resolution (or failure to resolve). Further recruitment and activation of inflammatory cells including neutrophils occurs at least in part via LTB_4 and cys-LTs acting on BLT1 and CysLT1 receptors respectively. The novel finding of LTC_4S expression by epithelial cells in both BM and OA tissue provides a further cellular source of cys-LTs, while confirmation of ubiquitous LTA_4 hydrolase in both stromal and epithelial sites provides a mechanism for amplification of LTB_4 pathways. Infiltrated neutrophils may engage in transcellular synthesis and donate LTA_4 to epithelial cells for this to occur (Haeggstrom 2000). Activation of the G-protein coupled LTB_4 receptor 1 (BLT1) located to epithelial cells promotes cellular proliferation and survival. The finding of epithelial expression of BLT1 receptors may correlate with more advanced stage disease and correlation of BLT1 receptor expression with disease stage or prognosis would provide clarification. Other LTB_4 pathway enzymes may also be important, such as the increased expression of FLAP seen in breast cancer, with high levels in aggressive tumours correlating with reduced survival (Jiang et al. 2006)

Whether 5-LOX pathway proteins contribute to the pathogenesis of OA or arise as a consequence is not clear and requires further study. Exploring levels of known pro-inflammatory and angiogenesis factors, such as vascular endothelial growth factor, matrix metalloproteinases, and apoptosis regulatory genes, and correlating these with 5-LOX pathway proteins should provide insight. This could be achieved both *in vitro* and *in vivo* using developed animal models (rat and mouse) of BM and OA (Xu et al. 2000). Use of pathway inhibitors could explore the functional relationship of these pathways. Genetically altered mice, for example *ALOX5* knockouts, could provide interesting insights into the importance of the 5-LOX pathway on development of BM and OA, as it has for other cancers. This was highlighted recently by the demonstration that loss of *ALOX5* prevents development of chronic myeloid leukaemia in mice (Chen et al. 2009). Mice with deletion of FLAP, LTA_4H, LTC_4S and the BLT and CysLT receptor subtypes have also been generated and these could be useful in disentangling their roles in appropriate models.

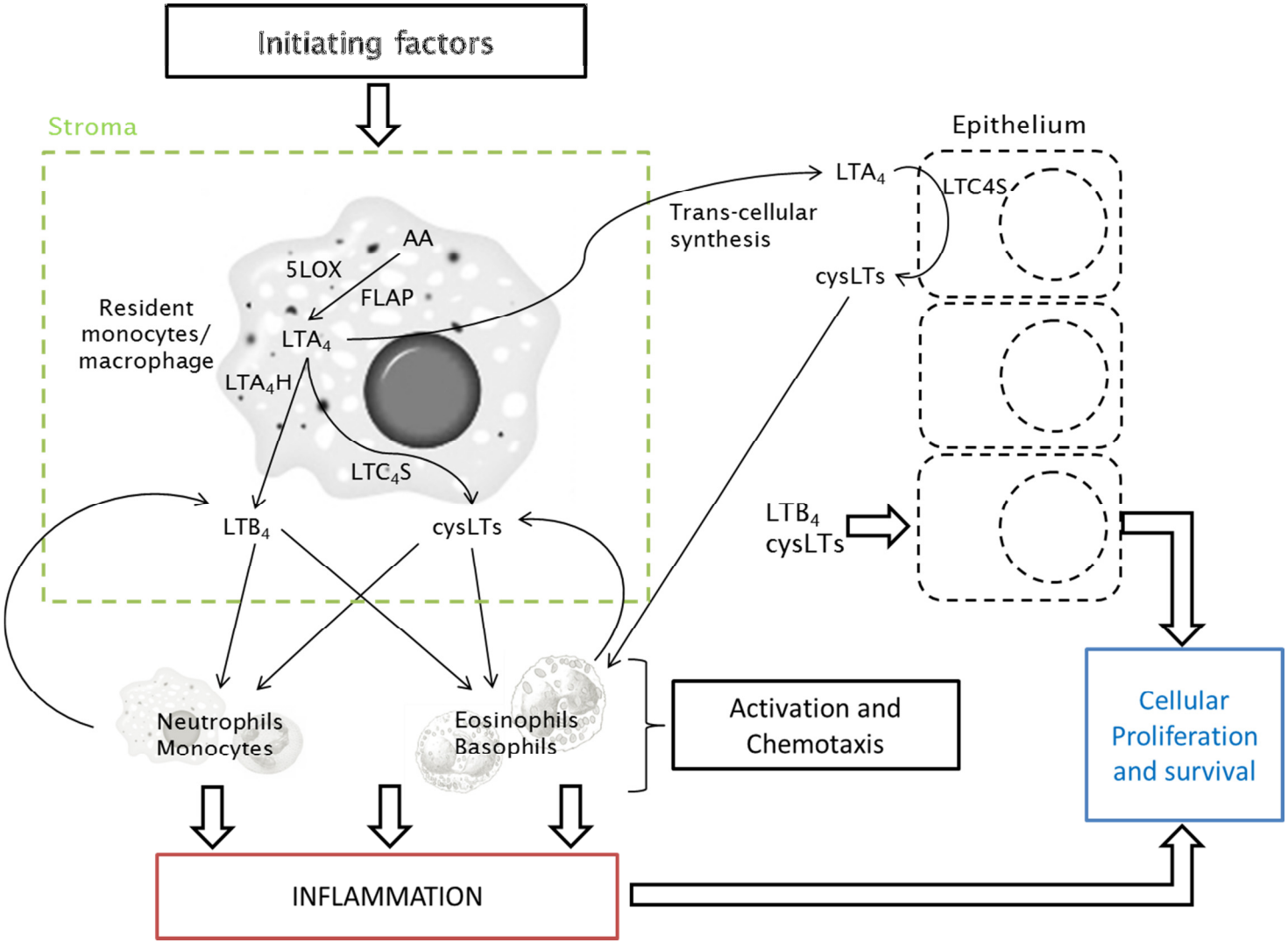


Figure 7-A: Illustration of the potential role of 5-LOX pathway in Barrett's adenocarcinoma

A full understanding of the role of eicosanoids in carcinogenesis requires unravelling of the many enzymatic pathways competing for substrate (see figure 7-B). A delicate balance exists between these pathways, with evidence for diversion down alternative pathways on selective inhibition. Exploring the preventative role of 5-LOX pathway inhibitors in BM with concomitant measurement of these pathways may provide novel insights. Inhibition of multiple or highly selective pathways may be required for an optimum chemo-preventative role, as there is a growing recognition of arachidonic acid as a source not only of inflammatory mediators such as leukotrienes and prostanoids, but also of anti-inflammatory, pro-apoptotic and pro-resolving lipid mediators derived from 15-LOX and other pathways (Serhan et al. 2008).

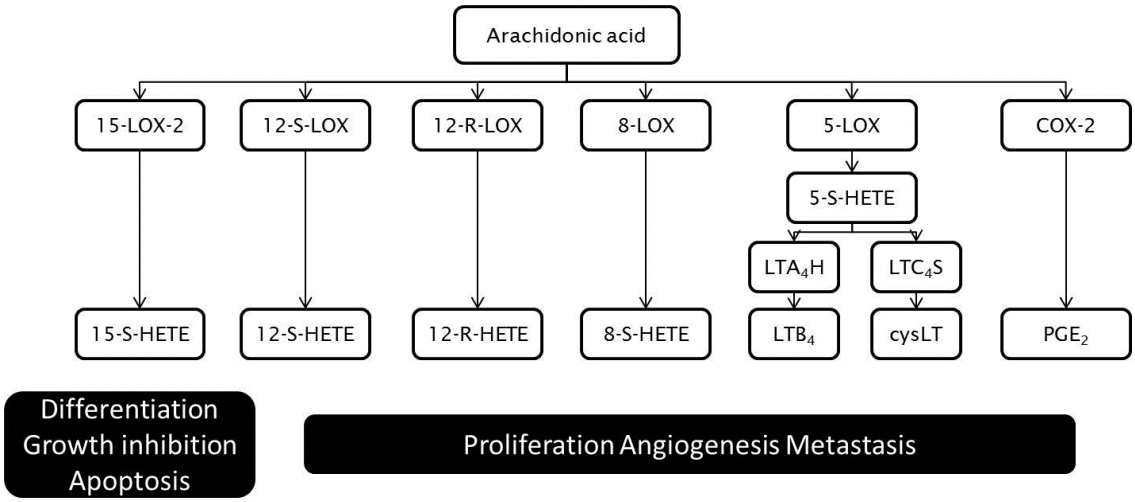


Figure 7-B: Metabolism of arachidonic acid by lipoxygenase and cyclo-oxygenase pathways and role in carcinogenesis

7.2 Endoscopic therapy for high risk patients with Barrett's oesophagus

Over the last decade emerging techniques have enabled endoscopic therapy for patients with Barrett's oesophagus at highest risk of developing invasive oesophageal adenocarcinoma. Over the last few years RFA using the HALO device has established itself as efficacious with minimal morbidity. The cost-effectiveness study described in this thesis and published elsewhere (see appendix G) has demonstrated that this technique may be performed cost-effectively in a UK setting when compared with the current "gold standard" of oesophagectomy.

The cost-effectiveness of RFA for patients with HGD or early non-invasive OA has now been demonstrated by a number of other independent studies (Inadomi et al. 2009, NICE 2010b, Pohl 2009). In this economic modelling, progression to cancer after treatment with RFA was an important determinant in the cost-effectiveness. After the publication of these studies, two year follow up data from the AIM-dysplasia trial has become available in the majority of patients (54/61) (Shaheen et al. 2011). The results demonstrate that 95% of patients who underwent RFA for HGD were still free of dysplasia. If translated to the health economic models (including that presented in this thesis), RFA would still remain a cost-effective strategy. However, the development of HGD or cancer in patients with an oesophagus apparently clear of intestinal metaplasia at 2 years post therapy, raises some cause for concern (Titi et al. 2012). 5-year outcome data should offer more robust conclusions regarding the cost-effectiveness of RFA.

Surveillance of non-dysplastic Barrett's oesophagus every 2-3 years is currently practised in the UK despite previous findings that this is a cost-ineffective approach when compared with no surveillance, and reduction in mortality is unproven (Garside et al. 2006). An on-going randomised controlled trial (BOSS study) hopes to ascertain whether surveillance helps to detect early cancers when compared with "at need" endoscopy. Notwithstanding the results of this trial, persuading patients with BM not to worry unduly about their increased risk of cancer may prove difficult. RFA of non-dysplastic Barrett's results in increased QALYs when compared with surveillance alone, but with a significant cost and, exceeding the NICE willingness to pay threshold of £20000-30000/QALY, and unknown efficacy (Das et al. 2009; Inadomi et al. 2009, NICE 2010b). Part of the reason behind this possible failure in a surveillance strategy is that the risk of development of OA in BM may have been previously overestimated, with most recent studies suggesting a much lower annual risk of 0.1-0.2%, compared with 0.5-1% in early studies (Hvid-Jensen et al. 2011; Bhat et al. 2011). The incidence of oesophageal cancer however continues to rise and mortality from the disease remains largely unchanged. Identifying patients with BM at highest risk in progressing to OA is therefore of paramount importance.

Current philosophy relies on the detection of dysplasia, and whilst the treatment of HGD appears rational, the treatment of LGD is less certain. Recent studies have demonstrated that LGD does confer an increased risk of developing HGD or OA of approximately 1.2-1.4% per annum (Hvid-Jensen 2011). The use of other biomarkers can however improve this risk stratification and future study should explore whether use of these other factors can determine the optimum cost-effective strategy for patients with non-dysplastic Barrett's. Promising panels of biomarkers are in development which could be utilised with recent study demonstrating an advantage using a panel of 3 biomarkers (LGD, abnormal DNA ploidy, and *Aspergillus oryzae*

lectin) when compared with using LGD, age and sex alone (Lieberman-Bird et al. 2012). A future strategy may involve treating those identified at highest risk of progression with RFA, whilst reassuring those with minimal or no risk and prevent costly surveillance, also shown to impact negatively on patients quality of life. This becomes especially paramount given the potential introduction of population screening for BM using the Cytosponge (Kadri et al. 2010). In addition, further understanding of the lipoxygenase's in the development of OA may allow innovative new chemo-preventative strategies or biomarkers to be developed.

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Dr CPC Boger

Appendix A – Ethical approval

NHS
National Research Ethics Service
ISLE OF WIGHT, PORTSMOUTH & SOUTH EAST HAMPSHIRE
RESEARCH ETHICS COMMITTEE

1ST Floor, Regents Park Surgery
Park Street, Shirley
Southampton
Hampshire
SO16 4RJ

Tel: 023 8036 2863
Fax: 023 8036 4110

Email: scsha.SEHREC@nhs.net

MFK

28 January 2008

Dr C Boger
Clinical Research Fellow in Gastroenterology
Southampton Hospitals NHS Trust
MP825
Level F
Southampton
SO16 6YD

Dear Dr Boger

Full title of study: **Evaluation of 5 lipoxygenase pathway expression, intracellular localisation, and quantification of mRNA levels in Barrett's metaplasia, dysplasia, and adenocarcinoma.**

REC reference number: **08/H0501/3**

The Research Ethics Committee reviewed the above application at the meeting held on 18 January 2008. Thank you for attending to discuss the study.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to submit the Site-Specific Information Form to any Research Ethics Committee. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Appendix B – Consent form and patient information sheet

Southampton NHS
University Hospitals NHS Trust

Medicine and Elderly Care
Department of Luminal Gastroenterology
Level F, Centre Block, Mail point CF91
Southampton General Hospital
Tremona Road
Southampton SO16 6YD

Tel: 023 80798462
Fax: 023 80794983

15/10/07 (Version 1)

CONSENT FORM

1 for patient, 1 for researcher, 1 for hospital notes

Title of the project: **Donation of biopsy/tissue during gastroscopy for research in understanding the progression of Barrett's oesophagus**

Name of researcher: Dr P Boger

PART A.

1. I have read the attached information sheet on the above project dated 15/10/07 and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and understand any foreseeable risks involved. ☐
2. I understand that my participation is voluntary and that I am free to withdraw my approval at any time without giving a reason and without my medical care or legal rights being affected. ☐
3. I give permission for my medical records to be looked at and information taken from them to be analysed in strict confidence by responsible people involved in this research project. ☐
4. I understand that (my doctor and/or I) will be informed if any of the results of the medical tests done as part of the research are important for my health. ☐
5. I agree to take part in the above study to collect a sample of the lining of my gullet. ☐

SAMPLES GIFTED FOR STORAGE AND USE IN FUTURE STUDIES

PART B. Linked Annonymised Samples

6. Provided that specific study protocols have been reviewed and approved by the Local Research Ethics Committee, I indicate my consent for the sample and its derived cells to be stored (potentially for many years) for the following types of studies. I understand that these studies are not for the purpose directly benefiting my health.

a) I give permission for the sample to be used for treatments/investigations of medical conditions relating to Barrett's oesophagus. ☐

b) I give permission for the sample to be stored for use in other unrelated research studies the precise nature of which will depend upon future scientific advances, but excluding genetic studies and germ-line research. ☐

c) I want / do not want (*delete as applicable*) to be told the results of this test. I understand I can change my mind about this later. ☐

d) In the case of linked annonymised samples, I give permission for a member of the research team to look at my medical records, to obtain further information about my medical condition. I understand that the information will be kept confidential. ☐

PART C. Unlinked Annonymised Samples

6. Provided that specific study protocols have been reviewed and approved by the Local Research Ethics Committee, I indicate my consent for the sample and its derived cells to be stored (potentially for many years) for the following types of studies. I understand that these studies are not for the purpose directly benefiting my health.

a) I give permission for the sample to be used for treatments/investigations of medical conditions relating to Barrett's oesophagus. ☐

b) I give permission for the sample to be stored for use in other unrelated research studies the precise nature of which will depend upon future scientific advances, but excluding genetic studies and germ-line research. ☐

c) I understand that future research, using the sample I give may include genetic research aimed at understanding the genetic influences on the development and progression of Barrett's oesophagus. ☐

d) I understand that the sample may be used for commercial development, without financial or other benefit to myself, for the investigation and treatment of medical conditions, potentially leading to new preventative measures against such conditions in keeping with the gift nature of my sample. ☐

_____	_____	_____
Patient name	Date	Signature
_____	_____	_____
Consenter (if not researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Southampton NHS
University Hospitals NHS Trust

Medicine and Elderly Care
Department of Luminal Gastroenterology
Level F, Centre Block, Mail point CF91
Southampton General Hospital
Tremona Road
Southampton SO16 6YD

Tel: 023 80796872
Fax: 023 80794983

15/10/07

Patient Information Sheet (Version 1)

Dear patient,

Title of the project: **Donation of biopsy/tissue during gastroscopy for research in understanding the progression of Barrett's oesophagus**

You are being invited to take part in a research study. This information sheet will help you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives, or your doctor/consultant if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you.

1. What is the purpose of this study?
We are conducting a study in order to better understand the role of certain enzymes (biological markers) in Barrett's oesophagus (inner lining of the gullet). A minority of patients with Barrett's oesophagus can develop further changes in this inner lining of their gullet. We are investigating the role of certain enzymes, which could explain why some patients with Barrett's oesophagus develop further changes in the inner lining of their gullet. For this research, we would like to take some additional samples (biopsies) during your planned gastroscopy. Your help by participating in this study will be greatly appreciated.

2. Why have I been chosen?
You have Barrett's or related condition and are attending for gastroscopy (your consultant will have explained this to you) for clinical reasons. For our research project we are collecting **six extra biopsies** from patients with Barrett's related conditions and your help will be appreciated.

3. Do I have to take part?
You don't have to take part in this study as participation is entirely on a voluntary basis and it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. Furthermore, you are still free to withdraw at any time without giving a reason. This will not affect the standard of care you receive.

4. What will happen to me if I take part?
You will have your gastroscopy procedure as planned by your consultant/doctor. During the procedure we will take six additional biopsies from your gullet. This will not cause any extra pain

or discomfort but **might prolong your procedure by a minute**. This will not affect your treatment in any way and does not involve any other tests, follow-up visits or interviews.

5. What do I have to do?
Taking part in his study does not involve any extra dietary, drugs or lifestyle restrictions. You should follow the standard advice given to you before and after gastroscopy.

6. What are the possible disadvantages and risks of taking part?
There are no extra disadvantages or risks over and above that for gastroscopy, which you are having for clinical reasons.

7. What are the possible benefits of taking part?
There are no immediate benefits to you but this research might help to improve the management of this condition in the future.

8. What if new information becomes available?
Sometimes during the course of a research project, new information becomes available. If this happens, your research doctor will tell you and your doctor about it.

9. What if something goes wrong?
It is difficult to foresee any harm resulting from this study. If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action, but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the National Health Service complaints mechanisms may be able to help you.

10. Will my taking part in this study be kept confidential?
All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital/laboratory will have your name and address removed so that you cannot be recognized from it.

11. What will happen to the results of the study?
We intend to have the study published in a relevant medical or professional journal. While sometimes this attracts attention form journalists, you will never be identified in any report or publication. There are no declared interests from any commercial company.

12. What will happen to the biopsies/tissue samples after the study?
With your permission (Consent form part C) we would like to store the remaining tissue samples in an unlinked anonymised manner to use in possible future approved research. This means that the tissue samples will be given a unique code number and stored for future research. With this code number it will be possible for the researcher to identify the samples but not you or your personal details.

13. Who is funding the research?
This research is being partly funded by a regional charity (Wessex Cancer Trust) but the doctor conducting the research is not being paid for including and looking after the patient in this study.

14. Who has reviewed the study?
The Isle of Wight, Portsmouth and Southeast Hampshire Research Ethics Committee have reviewed this study.

15. Contacts for further information
You may contact the research doctor, Dr P Boger directly on the telephone number given at the head of this information sheet, or discuss any aspect of the study with your doctor.

THANKYOU

You may keep a copy of this information sheet and the consent form you have signed.

Appendix C - Solutions/laboratory protocols

Tris Buffered Saline (pH 7.6)

Sodium Chloride	80g
Tris	6.05g
1M Hydrochloric Acid	38mls
Distilled water	10L

Mix buffer salts and acid in 1L of distilled water, adjust pH to 7.65 and add to remaining 9L of water to give a final pH of 7.6.

Processing of tissue into Glycol Methacrylate (GMA)

Steps 4 through to 7 of this procedure must carried out under a fume extraction hood.

1. Place biopsy immediately into ice cold acetone containing 2mM phenyl methyl sulphonyl fluoride (35mg/100ml, 175mg/500ml) and 20mM iodoacetamide (370mg/100ml, 1.85g/500ml).
2. Fix overnight at -20^{o}C.
3. Replace fixative with acetone at room temperature, 15 mins.
4. Methyl benzoate at room temperature, 15 mins.
5. Infiltrate with processing solution:5% methyl benzoate in glycol methacrylate (GMA solution A) at 4^{o}C, 3 x 2 hours
6. Prepared embedding solution: GMA solution A 10mls
   benzoyl peroxide 70mg

dissolve the benzoyl peroxide in solution A by gentle shaking, when dissolved add GMA solution B 250µl

7. Embed specimens in freshly prepared embedding solution in Taab flat bottomed capsules, placing biopsy in the bottom of the capsule and filling to the brim with resin and closing lid to exclude air
8. Polymerise at 4^{o}C for 48 hours
9. Store in airtight boxes at -20^{o}C.

White cell isolation for RT-QPCR

1. 6mls of acetyl citrate dextran solution to collection tube
2. Take blood, add to tube
3. Add 15mls dextran (6%) to 30mls blood
4. Allow to sediment at room temperature for 60minutes (with lid turned upside down to prevent top bubble formation), until clear interface between blood and plasma
5. Remove plasma with 25ml pipette and place in fresh tube. Discard red cells
6. Spin plasma @ 1150 x 12min at RT
7. Remove supernatant
8. Add TRIzol.

Appendix D – search strategies for parameters used in model

Oesophagectomy for HGD

Search strategy

Population - patients with HGD undergoing oesophagectomy

Intervention - oesophagectomy

Comparator - No oesophagectomy or other treatment modality i.e. PDT

Outcome - Morbidity, Operative mortality, Post-operative progression to adenocarcinoma, 5-year survival

Search terms - (high grade dysplasia OR dysplasia OR Barrett's oesophagus OR intestinal metaplasia) AND (oesophagectomy) AND (mortality OR morbidity OR complication OR survival OR adenocarcinoma)

Progression to adenocarcinoma post oesophagectomy after surgery for post RFA recurrence

Search strategy

Population - patients with OA undergoing oesophagectomy

Intervention - oesophagectomy

Comparator - No oesophagectomy or other treatment modality i.e. PDT

Outcome – Mortality, 5-year survival

Search terms - (oesophagus) AND (adenocarcinoma) AND (oesophagectomy) AND (mortality OR survival)

RFA for HGD

Parameters required:

Outcomes after RFA (Squamous lined oesophagus, Barrett's oesophagus without HGD, HGD or OA)

RFA complications (no complications, perforation, stricture requiring dilatation)

Search strategy

Population - Patients with HGD in Barrett's oesophagus

Intervention - oesophagectomy

Comparator - No oesophagectomy or other treatment modality i.e. PDT

Outcome – Removal of HGD, removal of Barrett's, post ablation progression to HGD or oesophageal adenocarcinoma, morbidity, procedural mortality, 5-year survival

Search terms - (high grade dysplasia OR dysplasia OR Barrett's oesophagus OR intestinal metaplasia) AND (Radiofrequency ablation OR ablation OR HALO) AND (mortality OR morbidity OR complication OR survival OR adenocarcinoma)
Mortality rate after oesophageal perforation or dilatation for oesophageal stricture

Mortality from other endoscopic procedures
Parameters required:
i) Death from iatrogenic oesophageal perforation
ii) Death from oesophageal dilatation

Search strategy
Population - Patients undergoing endoscopic dilatation of a benign oesophageal stricture or patients who have suffered a oesophageal perforation after an upper gastrointestinal endoscopy
Intervention - upper gastrointestinal endoscopy, oesophageal dilatation
Comparator - no dilatation or best supportive care
Outcome – perforation of oesophagus, procedural mortality

Search terms - (oesophageal dilatation OR oesophageal perforation) AND (endoscopy) AND (mortality OR morbidity OR survival)

Utility of oesophagectomy health states
A search strategy of (oesophagectomy) AND (quality of life OR utility OR utilities) identified.

Utility of various Barrett's disease states
A search strategy of (Barrett's) AND (quality of life OR utility OR utilities).

Appendix E - Utilities

Post oesophagectomy health states

The model cycle was 6-months although the median length of hospital stay is 10 days after an oesophagectomy, followed by a recovery phase at home. Utility values for the oesophagectomy health states were therefore calculated to reflect this as follows:

For 10 days whilst in hospital - utility of oesophagectomy without complications = 0.9, or utility of oesophagectomy with complications = 0.82

Followed by a utility of 0.92, to represent the recovering at home phase for the rest of that cycle.

Therefore utility of oesophagectomy without complication
= ((1/18) * 0.9) + ((17/18) * 0.92)
= 0.92

And utility of oesophagectomy with complications
= ((1/18) * 0.82) + ((17/18) * 0.92)
= 0.91

Appendix F – Costs used in the model

Radiofrequency ablation

The table below summarises the calculations for the RFA costs.

	Number of units	Cost per unit* (£)	Total (£)
Fixed			
Purchase HALO 360 generator	1	21500	21500
Purchase HALO 90 generator	1	11000	11000
			32500
Annual equivalent cost (1/7th)**	0.14	32500	4643
Variable			
HALO 360 catheter	1	985	985
HALO 90 catheter	2	650	1300
Sizing balloon	1	320	320
Endoscopy unit staff/time and other running costs (HRG code FZ03A)	3	406	1218
Total variable cost for 100 patients			382300
Total (fixed and variable) cost for 100 patients			386943
Average cost per patient over 1st year			3869.43

*all costs discounted at 3.5% per annum, **generators estimated by manufacturer to last 7 years total.

Oesophagectomy

The cost code that best matches uncomplicated oesophagectomy is FZ01B - Complex Oesophageal Procedures 19 years and over without CC at £6342 per elective procedure. The cost code that best matches complicated oesophagectomy is FZ01A - Complex Oesophageal Procedures 19 years and over with CC at £9305 per elective procedure.

Patients who require an oesophagectomy after developing a perforation from RFA will require a non-elective oesophagectomy i.e. emergency. There is an additional cost for performing a non-elective oesophagectomy in the National tariff system. In the model, the full cost of an oesophagectomy is accrued as the patient moves into the

complicated oesophagectomy health state. This additional cost is therefore calculated as:

£11924.00 (HRG code FZ01A Non-elective oesophagectomy with cc) - £9305.00 (FZ01A Elective oesophagectomy with cc FZ01A)

Endoscopic procedures

During the post RFA surveillance schedule patients require a routine gastroscopy with taking of quadrantic biopsies every 2cm. The cost code that best matches this is FZ03A - Diagnostic and intermediate procedures on the upper GI tract 19 years and over, planned same day tariff at £407 per procedure.

Oesophageal dilatation is performed endoscopically as a day case procedure. The cost code that best matches this is FZ25A - Therapeutic Endoscopic or Intermediate Stomach or Duodenum Procedures 19 years and over, planned same day tariff at £427 per procedure.

Post oesophagectomy care

Follow up post oesophagectomy was based on policy at Southampton University Hospital trust where patients are followed up after oesophagectomy as follows;
3-monthly during year 1, 6-monthly during years 2 and 3, annual years 4 and 5, then discharge from follow up if well.
Each outpatient visit accrues a cost, and the HRG code that best matches is 100 – general surgery outpatients at £72 per visit. This cost is subject to discounting at 3.5% per annum, and thus cost of outpatient visits in year 2 is £69.48, year 3 - £67.05, year 4 - £64.07, and year 5 – 62.44.

Palliation of untreatable adenocarcinoma

Costs were based on those previously estimated by Garside *et al.* 2006. We have thus allowed £1000 for GP and nursing costs, placement of an oesophageal stent, and 4 days respite care in hospital. The cost code that best matching oesophageal stent insertion is FZ24C - Major Therapeutic Open or Endoscopic Procedures 19 years and over without CC at £1379 per procedure. The cost code best matching respite care is WA15U - Respite care length of stay 4 days or less without CC at 1484 per stay. This gives a total cost of £3863 per patient for palliation of untreatable adenocarcinoma.

Appendix G - Full publications arising from thesis

Boger P.C., Turner D., Roderick P., Patel P. (2010). A UK-based cost-utility analysis of radiofrequency ablation or oesophagectomy for the management of high-grade dysplasia in Barrett's oesophagus. *Alimentary Pharmacology & Therapeutics* **32**(11-12): 1332-1342.

Boger P.C., Shutt, J. D., Neale J. R., Wilson S. J., Bateman A. C., Holloway J. W., Patel P., Sampson A. P. Increased expression of the 5-lipoxygenase pathway and its cellular localization in Barrett's adenocarcinoma. *Histopathology* **61**(3): 509-17.

AP&T Alimentary Pharmacology and Therapeutics

A UK-based cost–utility analysis of radiofrequency ablation or oesophagectomy for the management of high-grade dysplasia in Barrett's oesophagus

P. C. Boger*, D. Turner†, P. Roderick‡ & P. Patel*

*Department of Luminal Gastroenterology, MP CF91, Level F Centre Block, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, UK.
†Wessex Institute University of Southampton, Alpha House, Enterprise Rd, Southampton Science Park, Chilworth, Southampton SO16 7NS, UK.
‡Public Health Sciences and Medical Statistics, C Floor, South Academic Block, Southampton General Hospital, University of Southampton, Tremona Road, Southampton SO16 6YD, UK.

Correspondence to:
Dr P. C. Boger, Department of Luminal Gastroenterology, MP CF91, Level F Centre Block, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, UK.
E-mail: philboger@btinternet.com

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SUMMARY

Background
In the UK, oesophagectomy is the current recommendation for patients with persistent high-grade dysplasia in Barrett's oesophagus. Radiofrequency ablation is an alternative new technology with promising early trial results.

Aim
To undertake a cost–utility analysis comparing these two strategies.

Methods
We constructed a Markov model to simulate the natural history of a cohort of patients with high-grade dysplasia in Barrett's oesophagus undergoing one of two treatment options: (i) oesophagectomy or (ii) radiofrequency ablation followed by endoscopic surveillance with oesophagectomy for high-grade dysplasia recurrence or persistence.

Results
In the base case analysis, radiofrequency ablation dominated as it generated 0.4 extra quality of life years at a cost saving of £1902. For oesophagectomy to be the most cost-effective option, it required a radiofrequency ablation treatment failure rate (high-grade dysplasia persistence or progression to cancer) of >44%, or an annual risk of high-grade dysplasia recurrence or progression to cancer in the ablated oesophagus of >15% per annum. There was an 85% probability that radiofrequency ablation remained cost-effective at the NICE willingness to pay threshold range of £20 000–30 000.

Conclusion
Radiofrequency ablation is likely to be a cost-effective option for high-grade dysplasia in Barrett's oesophagus in the UK.

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INTRODUCTION

Barrett's oesophagus (BO) is estimated to be prevalent in 0.5–2% of adults in the western world, rising to approximately 15% in those with chronic gastro-oesophageal reflux disease.[1–3] The main complication is the development of oesophageal adenocarcinoma (ADC), the incidence of which is highest in the UK and rising faster than any other solid tumour in western countries.[4, 5] Once invasive cancer develops, the outlook is poor, with a 5-year survival of only 10–13%.[6, 7] In an attempt to preempt this, it is currently recommended that patients with BO undergo surveillance endoscopy every 2–3 years. Detection of premalignant or dysplastic change on biopsy signals an increased risk of malignant development, especially high-grade dysplasia (HGD), where the risk of progression to invasive cancer is 30–35% within 5 years.[8]

Current UK guidance recommends that for patients with persistent HGD despite intensive acid suppression, oesophagectomy be performed in a specialist centre in those fit for surgery.[9] However, surgical oesophagectomy is a high-risk procedure, even in tertiary centres, with a 30-day mortality rate of 3.2%, significant post-operative complications in up to 40%, and long-term morbidity.[10, 11] Oesophagectomy is expensive, with an average cost to the NHS of approximately £7500/person.

A number of alternative less invasive ablative therapies exist that aim to remove the dysplastic Barrett's epithelium and facilitate the restoration of normal squamous epithelium. Photodynamic therapy (PDT), approved by the National Institute for Clinical Excellence (NICE) for treatment of HGD in BO, involves intravenous administration of a photosensitizing agent, followed by endoscopic light laser exposure to the Barrett's segment.[12] PDT is effective in eliminating HGD in BO.[13] However, widespread acceptance of the technique has been held back by treatment-related adverse events in up to 94% and a high incidence of oesophageal strictures in up to 36%.[14]

Radiofrequency ablation (RFA) using the HALO device is an alternative new management strategy for patients with HGD in BO. Recent results from a randomized sham controlled study have shown that it is effective in eliminating HGD in BO in up to 90% of patients with complete reversion to squamous oesophageal lining in 80% at 12-month follow-up, and minimum treatment-related side effects.[15] The cost per patient to the NHS is not currently known, and there has been no randomized controlled trial comparing RFA and oesophagectomy.

The aim of this study was to model whether RFA followed by oesophagectomy for persistence or recurrence of HGD is a cost-effective option for the treatment of BO with HGD when compared with the current UK recommendation of oesophagectomy, and to identify critical parameters to guide further research.

METHOD

Patients

Our model consisted of a hypothetical cohort of 100 male patients aged 64 years, the mean age of patients undergoing oesophagectomy for HGD in published trials over the last 10 years.[16–26] We assumed the following pretreatment assessment for both strategies: endoscopy had been undertaken on two occasions revealing BO with HGD, and any visible nodules, masses or surface irregularities had been removed by endoscopic mucosal resection. Patients found to have invasive cancer after endoscopic mucosal resection were excluded from the analysis. It is assumed that patients were fit for both treatment modalities. The model included patients with either uni- or multifocal HGD.

The model

A Markov model was constructed in Microsoft Excel to simulate the two treatment strategies; RFA with surveillance and oesophagectomy for HGD or ADC recurrence/persistence, or immediate oesophagectomy. Figures 1 and 2 summarize the structure of the treatment

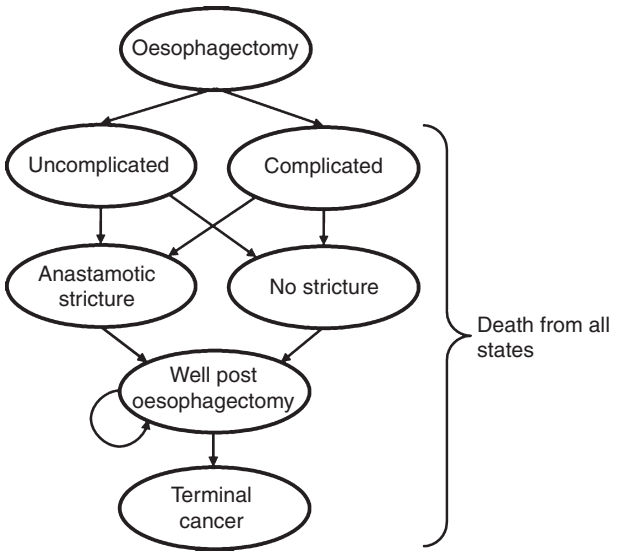

Figure 1 | Transition state diagram of oesophagectomy treatment strategy.

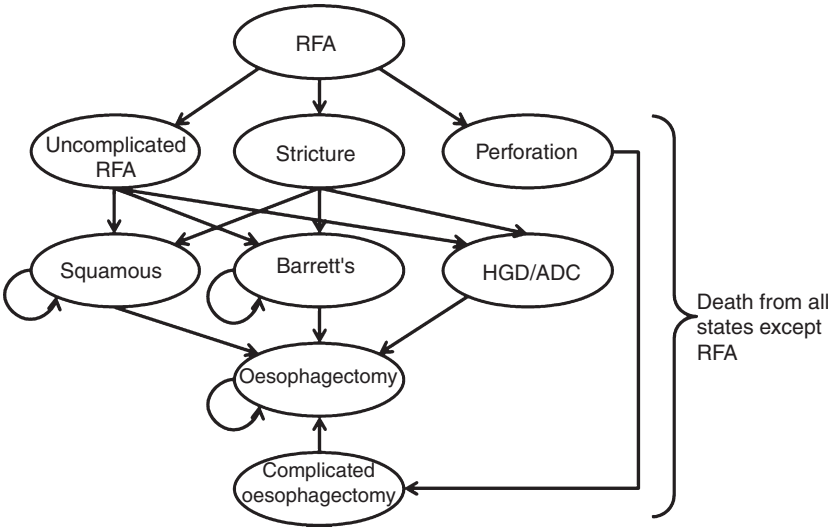

Figure 2 | Transition state diagram of radiofrequency ablation (RFA) treatment strategy.

strategies. The model was constructed to include major procedural and postprocedural complications and mortality reported in the literature. At the end of each cycle, which corresponded to 6 months, patients would progress to a health state depending on the derived transition probabilities. UK life table mortality rates were incorporated into the model to account for age-specific mortality from other causes.[27] Utility and cost were attributed to each state, which patients would accumulate as they progressed through the model. The study followed up the cohort for 25 years or until death. We estimated costs from an NHS perspective and outcomes from the viewpoint of the patient. All costs and benefits were discounted at 3.5% as recommended by the NICE.[28]

Treatment strategies and model assumptions

Oesophagectomy. In this strategy, patients with confirmed HGD in BO underwent immediate oesophagectomy. If a patient developed ADC post-operatively, it was assumed that he entered a 6-month terminal cancer phase, representing the median survival time with inoperable oesophageal cancer, and allowing the modelling of costs and measures of quality of life experienced by this group of patients, such as palliation of dysphagia with oesophageal stenting.[29] If a patient did not progress to terminal cancer during the first 5-years postoesophagectomy, he was considered cured. All patients were followed up in outpatient clinics post-oesophagectomy for 5 years as follows: 3-monthly for year 1, 6-monthly for the years 2 and 3, and then annually for years 4 and 5.

Radiofrequency ablation. In this strategy, patients with confirmed HGD in BO underwent a treatment course of RFA with the HALO device (Barrx Medical, Sunnyvale, CA, USA). This is delivered endoscopically under conscious sedation as a day-case procedure. The HALO 360 is a balloon-based device that is able to deliver a circumferential radiofrequency burn to the mucosal lining of the oesophagus, whereas the HALO 90 allows targeting of mucosa over an approximate 90° radius. Published experience to date suggests an average of two HALO 360, and two HALO 90 sessions are needed per patient to achieve complete removal of the Barrett's segment and replace it with a neo-squamous lining.[30]

There is no fixed surveillance strategy for patients with neo-squamous and BO post-RFA. For the base case analysis, we assumed the following strategy. If neo-squamous oesophagus is found after RFA, then yearly endoscopy was performed for 5 years, and if free of BO or worse pathology after that time, no further endoscopic follow-up was undertaken. If the patient had BO after RFA treatment, yearly endoscopy was performed for the first 5-years, and if free of HGD or worse at that time, endoscopic surveillance continued on a 2-yearly basis as per current UK practice. In the scenario analysis, this screening protocol was altered to analyse what effect it would have on the outcome of our cohort.

If HGD or ADC is picked up during the above surveillance strategies, the patient undergoes an oesophagectomy. This part of the RFA model has the same structure as the oesophagectomy strategy. We assumed that the risk of developing cancer in the neo-squamous oesophagus is most probable during the first 5 years post-treatment, and thus after this period (as with the postoesophagectomy group), the patient was considered cured.

The risk of developing HGD or ADC in patients with neo-squamous oesophagus was estimated using published

data of having residual nonvisible islands of Barrett's, termed subsquamous intestinal metaplasia (IM).[15] The low value assumed no risk of residual subsquamous IM and the high value assumed a higher risk of having nonvisible islands of Barrett's found with previous ablative therapies, and assuming that the residual Barrett's contained HGD.[31]

There are no data on the outcome of those with treatment failure post-RFA. For this, we have drawn parallels with data from other ablative therapies (PDT) where longer term follow-up is available. This demonstrates that treatment failures were still operable at time of progression, and revealed disease not extending beyond the submucosa (T1) on post-operative staging.[22] On this basis, in our model, those with post-RFA treatment failure had operable disease, but with a reduction in 5-year survival consistent with the predicted more advanced disease.

Search strategy for parameters

Parameter values for transition probabilities and utilities were obtained from peer reviewed literature. We searched the Medline database for the period from May 1999 to May 2009, as well as abstracts from Digestive Diseases week and British Society of Gastroenterology Annual Meeting. Bibliographic lists of relevant articles were searched, and in addition, we obtained a list of relevant papers from the manufacturers of the HALO device. We chose the most suitable study (studies) to provide the parameters for the base case, using data from randomized controlled trials, if available. If not available, we chose data from well-constructed observational (cohort) studies, with inclusion criteria similar to those in our hypothetical cohort. We assessed inclusion criteria, baseline assessment, size, duration of follow-up, loss to follow-up and assessment of outcome. In addition to the base case values, we selected a plausible range (high/low values) for use in the sensitivity analysis. This range was derived from variations in the published literature, or by author consensus when information was not available or there was considerable uncertainty in the literature. Annual risks and 5-year survival data were converted to 6-monthly probabilities using validated formulae,[32] with the assumption that 6-monthly progression rates were constant. Derived transition probabilities are shown in Table 1.

Utilities

Where available, we selected utilities derived using the standard gamble methodology, which avoids measurement biases associated with ratings scales, such as a visual analogue score.[39] We were unable to obtain utility values for all model states, and thus used known recorded values to provide a framework by which unknown values were estimated by consensus among the authors. Low and high values were derived either from 95% confidence intervals if available, or estimated by the authors to allow for the uncertainty surrounding the individual parameter. Derived utilities are shown in Table 2. Utility values were then used to generate quality adjusted life years (QALYs), which were used as the outcome measure in the model.

Costs

The costs used in the model are shown in Table 3. A majority of costs were estimated from NHS reference costs.[42] This derives costs from nationally collected data from NHS trusts and calculates an average total cost of delivering a treatment episode to a patient. The cost of RFA is not available on this national tariff and thus was estimated using direct and variable costs derived from information obtained from the UK distributor (Synetics Medical Limited, Middlesex, UK). All costs were for 2009/2010 and are shown in UK pounds sterling. For the sensitivity analysis, all cost were varied by $\pm 25\%$.

Sensitivity analysis

Deterministic. To enable thorough interrogation of the model and the input parameters, we initially undertook one-way sensitivity analysis, in which the various parameters in the model are varied throughout the specified range to assess how this impacts study results.[39] For areas where uncertainties in the literature were identified or to interrogate areas where assumptions had been made, we undertook threshold analysis, which allows the critical value of a parameter central to the decision to be identified.[39] Where uncertainty in the methodology of the model existed such as in the time period of surveillance post-RFA, a scenario analysis was undertaken to explore the effects of increasing surveillance.

Probabilistic. Probabilistic sensitivity analysis allows for all input parameters in a model to be specified as full probability distributions, rather than point estimates, allowing a representation of the uncertainty surrounding their values.[43] In the UK, it is a requirement for all cost effectiveness models submitted to NICE. The distributions and parameters used for each variable in the model are shown in Tables 1–3. For probabilities of an event either occurring or not occurring, we used

Table 1 | Transition probabilities

Description	Base case estimate	Range	Distribution	Source
RFA complications				
No complications	0.94	0.90–1	Jointly determined Dirichlet	[15, 33, 34]; assumption
Perforation	0.0005	0–0.005		
Stricture requiring dilatation	0.06	0–0.09		
Outcomes after RFA				
Neo-squamous lined oesophagus	0.82	0.45–0.91	Jointly determined Dirichlet	[15, 33, 34]; assumption
Barrett's oesophagus without HGD	0.11	0.09–0.25		
HGD or adenocarcinoma	0.08	0–0.30		
Progression probabilities during post-RFA surveillance (per cycle – 6 months)				
Neo-squamous to HGD/ADC	0.0002	0–0.0027	Beta (α = 1, β = 4596)	[31, 35, 36]
Barrett's to HGD/ADC	0.0068	0–0.0085	Beta (α = 4, β = 953)	
Oesophagectomy complications				
Peri-operative complications	0.37	0.27–0.57	Beta (α = 26, β = 44)	[18, 22, 25]
Anastamotic stricture	0.51	0.13–0.83	Beta (α = 27, β = 26)	[18, 22, 26]
Progression to adenocarcinoma postoesophagectomy				
After oesophagectomy for HGD	0.001	0–0.007	No distribution	[18, 22, 23, 25]
After oesophagectomy for post-RFA recurrence	0.0057	0–0.021	Beta (α = 18, β = 3239)	[22, 37]
Mortality				
Death from iatrogenic oesophageal perforation	0.35	0–0.5	Beta (α = 2, β = 15)	[38]
Death from oesophageal dilatation	0.005	0–0.01	Beta (α = 3, β = 551)	[38]
Death from oesophagectomy	0.014	0–0.045	Beta (α = 1, β = 69)	[22, 23, 25, 26, 37]

RFA, radiofrequency ablation; HGD, high-grade dysplasia; ADC, adenocarcinoma.

Table 2 | Health state utilities

Description	Base case estimate	Range	Distribution	Source
RFA without complications	0.94	0.84–1	Beta (α = 31, β = 2)	Assumptions based on reference [40] and [41]
RFA complicated by stricture	0.92	0.82–1	Beta (α = 31, β = 3)	
Post-RFA with neo-squamous oesophagus	0.97	0.9–1	Beta (α = 42, β = 1.3)	
Post-RFA with Barrett's oesophagus	0.95	0.883–1	Beta (α = 50, β = 3)	
Oesophagectomy without complications	0.92	0.77–1	Beta (α = 19, β = 2)	[40]
Oesophagectomy with complications	0.91	0.66–1	Beta (α = 9, β = 1)	
Anastamotic stricture post-oesophagectomy	0.92	0.841–0.999	Beta (α = 41, β = 4)	[41]
Fully recovered post-oesophagectomy	0.96	0.89–1	Beta (α = 46, β = 2)	[40]
Terminal oesophageal cancer	0.34	0.03–0.65	Beta (α = 3, β = 5)	

RFA, radiofrequency ablation.

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Table 3 | Costs

Cost estimate (£)	Base case*	Reference
Treatment course of RFA	3869	Costs supplied by Synetics Medical Ltd
Additional cost from developing perforation from RFA (excludes oesophagectomy)	2619	NHS reference costs 2009/2010 [42]
Routine endoscopy and biopsies	406	
Elective oesophagectomy	6342	
Oesophagectomy with complications	9305	
Oesophageal dilatation	427	
Outpatient follow-up per visit	72	
Palliation of untreatable adenocarcinoma	3863	[36], NHS reference costs 2009/2010 [42]

RFA, radiofrequency ablation.

* For the probabilistic sensitivity analysis, we used a gamma distribution on the assumption that ±25% formed a 95% confidence interval.

beta distributions, which generate values between 0 and 1 and hence avoid unfeasible values. As the method of generating utilities used was based on standard gamble, negative values were not possible, and hence the beta distribution is appropriate. Where more than two events are possible, Dirichlet distributions were chosen, which ensure that the total values of the probabilities of all events considered sum up to 1. Gamma distributions cannot take negative values and have a skewed distribution, which is characteristic of many forms of cost data.

We repeatedly ran the model 5000 times using values randomly drawn from these distributions. To illustrate the uncertainty relating to our cost effectiveness results, we calculated a cost effectiveness acceptability curve, which estimates the probability that an intervention is cost-effective at different values of a QALY.[44]

RESULTS

Base case

The results from the base case analysis are summarized in Table 4. For a 64-year-old male patient, a strategy of RFA followed by oesophagectomy, if there was disease recurrence, would be expected to cost £1902 less and result in 0.4 more QALYs, when compared with immediate

Table 4 | Base case results

Outcome per strategy	Oesophagectomy	RFA	Incremental ratio
Cost (£)	8555	6653	–1902
Unadjusted life-years	17.0	17.4	
QALY	13.8	14.2	0.4
Incremental cost effectiveness ratio (£/QALY)		Dominates	
Morbidity (%)			
Procedural	37.1	3.9	
Strictures	50.0	16.9	
Causes of death (% deaths)			
Age-related	96.4	99.1	
Surgical	1.9	0.2	
Endoscopic	0.7	0.2	
Cancer	1	0.5	

RFA, radiofrequency ablation; QALY, quality adjusted life year.

oesophagectomy. The RFA strategy thus dominates immediate oesophagectomy. In addition, the RFA strategy results in less morbidity (20.8% vs. 87.1%), procedure-related mortality (0.4% vs. 2.6%) and cancer deaths (0.5% vs. 1%) than immediate oesophagectomy.

Sensitivity analysis

The one-way sensitivity analysis demonstrated that the following were important factors in determining the cost effectiveness of RFA: outcome post-RFA, utility post-RFA with squamous oesophagus, and utility fully recovered post-oesophagectomy.

Only at the most pessimistic outcome post-RFA (i.e. 30% post-RFA with HGD), was RFA more costly than oesophagectomy, although it still resulted in more QALYs, costing £661/QALY. Threshold analysis demonstrated that for oesophagectomy to dominate as the preferred strategy, it required over 44% of patients to have residual HGD post-RFA.

We altered utility values highlighted by the one-way sensitivity analysis. For oesophagectomy to become a more effective (but still more costly) option, it would require either a utility value of <0.93 post-RFA with squamous oesophagus, or a utility fully recovered post-oesophagectomy of over 0.99. Thus, it would require fully recovered post-oesophagectomy to be a more preferable health state than post-RFA with squamous oesophagus.

As there is uncertainty in the robustness of the neo-squamous epithelium post-RFA, we undertook further threshold analysis to identify the HGD recurrence required to alter the results of the model. For this to occur, it would require an annual risk of HGD recurrence in the neo-squamous epithelium of 20%.

The need for or frequency of endoscopic surveillance after successful ablation with RFA is not currently known. We modelled a scenario of continuing surveillance in all patients post-RFA, with 2-yearly endoscopy for both neo-squamous and BO, after initial yearly endoscopy for the first 5 years. With this strategy, the cost of RFA increased considerably to £8168 with 14.2 QALYs. The cost of oesophagectomy remained at £8555 with 13.8 QALYs, and thus RFA still dominated as the preferred strategy.

As the method used to calculate the cost of RFA differed from other calculated costs, we undertook threshold analysis to determine how expensive treatment with RFA would need to be, to alter the outcome of the model. For RFA to become a more expensive treatment strategy, it would require an increase of £1904 from the base case estimate, to £5773 per treatment course per patient. RFA retained its cost effectiveness at a willingness to pay (WTP) of £30 000/QALY even if cost was increased to £17 754, i.e. 450% more than the base estimate and over twice the cost of an oesophagectomy without complications.

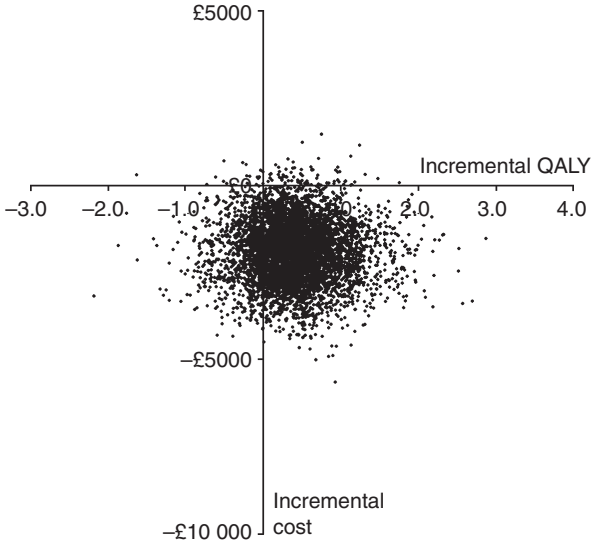

Figure 3 | Cost effectiveness plane of results of probabilistic sensitivity analysis.

Altering all other variables through their specified range, including age (55–70 years), and in addition altering the discount rate (1–5%), and the length of time the cohort was followed up for (5–30 years), made no critical difference to the model outcome.

Probabilistic sensitivity analysis

The cost effectiveness plane demonstrating the distribution of the results is shown in Figure 3. This scatter plot of the incremental costs and QALY pairs, generated from the probabilistic sensitivity analysis, illustrates the uncertainty surrounding the estimates of expected costs and expected effects associated with the RFA compared with the oesophagectomy strategy. A majority fall in the south-east quadrant, the position where the RFA strategy is more effective and less costly than the oesophagectomy strategy, and thus RFA is said to dominate.

The cost effectiveness acceptability curve shows the probability that RFA is cost-effective as the value placed upon a QALY is varied between £0 and £50 000 (Figure 4). The probability that RFA is cost-effective is approximately 84–86% throughout a WTP of £20 000–30 000/QALY, showing that RFA appears highly likely to be cost-effective.

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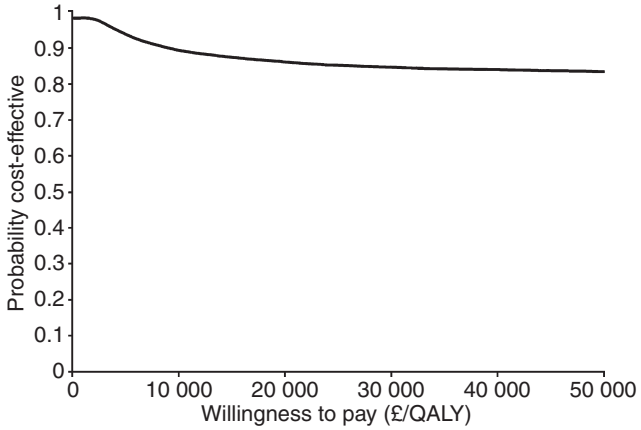

Figure 4 | Cost effectiveness acceptability curve for the strategy of radiofrequency ablation (RFA) compared with oesophagectomy.

DISCUSSION

Our study demonstrates that for a 64-year-old patient in the UK with HGD in BO, a treatment strategy of RFA followed by surveillance with oesophagectomy for HGD recurrence or progression to ADC is less expensive and results in more discounted QALYs compared with immediate oesophagectomy. Our study supports recent findings, which demonstrated that in a US setting, a strategy of RFA for HGD yielded more QALYs at less cost than surveillance, oesophagectomy or other ablative strategies, with the exception of PDT, which gave comparable QALYs, but at a higher cost.[45]

One of the roles of a health economic analysis such as ours is to identify areas of uncertainty, which may guide future study. Our model has identified the following key areas: the first concerns the success rate achievable by RFA in eliminating HGD from BO. Data thus far show that RFA is very effective at eliminating HGD with success in 90% or more of cases, and with removal of BO between 60% and 91%. The modelling demonstrated that RFA remains cost-effective even if eradication of HGD is achievable in only 60% of cases at a WTP of £30 000/QALY, a figure well below the 90–97% in published trials,[15, 33, 34, 46, 47] although the longest follow-up available is currently limited to a median of 2 years.[34]

Secondly, our one-way sensitivity analysis suggested that for oesophagectomy to become the preferred strategy, it would require the utility of patients post-RFA with neo-squamous oesophagus to be lower than utility of those post-oesophagectomy. Utility values for patients undergoing RFA have not yet been derived, although there is some preliminary evidence that patients with resolution of dysplasia post-RFA have an improvement in surrogate markers of quality of life, such as depression, stress and difficulty sleeping as measured using a visual analogue scale.[48] We used deliberately conservative estimates of utility based around known values ascertained by standard gamble technique, considered a robust method for deriving utility values.[39] Although minimally invasive techniques for oesophagectomy are improving, resulting in lower morbidity and mortality, it is unlikely that a surgical approach would be a favoured patient preference.[49]

A recent health economic evaluation by NICE looking at ablative therapies for treatment of HGD in BO has demonstrated that RFA with subsequent surveillance resulted in more QALYs, but was considerably more expensive than oesophagectomy.[50] This in part contrasts our findings where we found that although the cost of RFA increased considerably with surveillance, it was still cheaper than oesophagectomy with more QALYs, and thus still dominated oesophagectomy as the preferred strategy. We believe, the explanation for the lack of parity was the inclusion of the cost of proton pump inhibitors in their model in the post-RFA, but not in the post-oesophagectomy strategy. We would argue that this unfairly biases the study in favour of oesophagectomy for two reasons. First, published quality of life data have suggested that up to 75% of patients post-oesophagectomy still complain of reflux symptoms, and this would likely have a major impact on cost incurred for medication usage, and potentially on visits to their general practitioner or hospital consultations.[11] Secondly, in their model, no allowance has been made for poor compliance, which would lead to falsely high costs in the RFA strategy.

The need for or frequency of endoscopic surveillance after successful ablation with RFA is not currently known. The long-term stability of the neo-squamous epithelium is not yet determined and this is likely to lead to clinicians continuing surveillance on their patients, despite apparent histological resolution. In our model, although the cost of RFA strategy increased considerably due to follow-up and surveillance, it dominated the oesophagectomy strategy. In both the NICE health economic model and the published US study, surveillance considerably added to the cost of ablative therapies, and made RFA a more expensive strategy than oesophagectomy.[45, 50] The frequency and duration of follow-up endoscopy for patients who have undergone successful ablation will thus need to be considered very carefully,

once the durability of the neo-squamous mucosa has been determined with long-term follow-up data.

Other management strategies for treating HGD in BO exist such as surveillance alone, PDT or other ablative therapies. In the UK, surveillance of those with HGD is not presently practised. Several previous cost-effective analyses comparing ablative therapy with surveillance have found surveillance to be costly and inefficient in preventing progression to cancer.[45, 51, 52] Studies from the US have demonstrated that PDT is an effective alternative to oesophagectomy, but at a wide variation in cost between $6000 and $50 000/QALY. Acceptance of PDT as an option in the UK has been hampered by a stricture rate as high as 35%, and the ultimate progression to cancer in 15%.[13] RFA is a realistic option, given its low complication rate, and it appears to be effective in preventing cancer deaths, with terminal cancer the cause of death in only 0.5% of our hypothetical cohort. Currently, no patient in reported trials has progressed to inoperable cancer, but longer periods of follow-up are needed before this apparently low risk can be confirmed.

We have made a number of assumptions in our model which warrant further discussion. There is an established progression from Barrett's IM, through low-grade dysplasia (LGD), HGD to invasive ADC. For the purpose of this model, IM and LGD were grouped together. We have based this assumption on recent data, which have suggested that LGD did not appear to confer an increased risk of developing cancer.[35] In fact, a majority regressed to IM or went back and forth between LGD and IM. In addition, the histological classification and inter-observer agreement in the diagnosis of LGD is variable, with factors such as inflammation playing an important role in misclassification. Another assumption is not distinguishing between uni- and multifocal HGD, in accordance with the other economic analyses in this area.[45, 50] There is evidence to suggest that patients with multifocal HGD are more likely to have a high risk of progression to ADC compared with patients with unifocal HGD.[53] Even with this increased risk, it is felt unlikely that this would affect the outcome of our model, given the outcome of our threshold analysis. In addition, these findings were not supported by another retrospective study.[54] The parameters used in our model included patients with both histological subtypes, and thus one would expect allowance for the potential increase risk with multifocal HGD.

We have excluded those with T1 disease. Endoscopic resection of oesophageal ADC confined to the mucosa (T1a) is practised in the UK, given the low likelihood of lymph node invasion.[55] However, there is a high risk of recurrent disease, with cancer-free survival of 80% in one series (although these patients were able to have re-treatment endoscopically without influencing survival) compared to 97% of those undergoing an immediate oesophagectomy.[56] There are preliminary data suggesting that RFA of the remaining Barrett's segment after endoscopic resection of the T1a lesion can be achieved with favourable outcomes.[33] Follow-up was limited to a median of 21 months, during which one patient (of 16 in the cohort with ADC) developed disease infiltrating into the submucosa (stage T1b), which may be associated with lymphatic spread in 20%.[55] The quality of life, costs and outcomes involved in such a treatment strategy are complex, and the health economic argument is therefore best served by a separate analysis.

Randomized controlled trials are generally considered the most robust method of determining the benefit of a new treatment,[57] and certainly one could conclude that a randomized control trial of oesophagectomy vs. RFA would be the next logical step. However, randomized control trials are costly and time consuming. The results of the model support the case that RFA should be the optimum strategy, and that only with pessimistic parameters does this outcome change. However, it may be influenced by the outcome after RFA, and thus prospective long-term outcome data from a well constructed clinical trial, with population-matched controls, such as the ongoing Ablation of Intestinal Metaplasia (AIM)-dysplasia sham-controlled trial in the US, should be sufficient to confirm that RFA is the most (cost-)effective strategy. The critical issue is if clinicians have equipoise regarding the two strategies, despite the findings of the modelling, a definitive trial is necessary.

In conclusion, our study has suggested that a strategy of RFA with oesophagectomy for HGD recurrence is a cost-effective strategy when compared with immediate oesophagectomy for the management of BO with HGD in the UK. Sensitivity analysis demonstrates that RFA remains the preferred option providing removal of HGD in BO is achieved in over 60%. Long-term prospective outcome data will help clarify whether RFA should become the first line treatment option for HGD in BO in the UK.

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Increased expression of the 5-lipoxygenase pathway and its cellular localization in Barrett's adenocarcinoma

Philip C Boger, James D Shutt, James R Neale, Susan J Wilson,[1] Adrian C Bateman,[2] John W Holloway,[3] Praful Patel & Anthony P Sampson[3]

Department of Luminal Gastroenterology, University Hospital Southampton NHS Foundation Trust, Southampton General Hospital, [1]Histochemistry Research Unit, University of Southampton Faculty of Medicine, [2]Department of Cellular Pathology, University Hospital Southampton NHS Foundation Trust, Southampton General Hospital, and [3]Clinical & Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, UK

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Increased expression of the 5-lipoxygenase pathway and its cellular localization in Barrett's adenocarcinoma

Aims: Up-regulation of the 5-lipoxygenase (5-LOX) leukotriene pathway is evident in numerous tumour types, and has been linked to the promotion of cancer cell growth. The aim of this study was to evaluate the immunohistochemical expression of 5-LOX pathway proteins in oesophageal adenocarcinoma and its pre-malignant lesion, Barrett's metaplasia.

Methods and results: Tissue samples were collected at endoscopy from 16 patients with Barrett's metaplasia and from seven with oesophageal adenocarcinoma; five proximal squamous oesophagus samples were used as controls. Immunohistochemical analyses were performed on stromal and epithelial areas with optimized concentrations of primary antibodies for 5-LOX, 5-LOX-activating protein (FLAP), and the distal enzymes leukotriene (LT) A_4 hydrolase (LTA_4H) and LTC_4 synthase (LTC_4S). the diagnosis was histologically confirmed from adjacent sections by a gastrointestinal pathologist. Striking increases in the stromal immunoexpression of 5-LOX ($P = 0.041$), FLAP ($P = 0.038$), LTA_4H ($P = 0.0008$) and LTC_4S ($P = 0.036$) were seen in adenocarcinoma tissue. Stromal FLAP and LTA_4H immunostaining correlated with elevated neutrophil counts ($P < 0.001$). LTC_4S was also notably overexpressed within epithelial cells in both Barrett's metaplasia ($P < 0.001$) and adenocarcinoma ($P < 0.01$) tissue.

Conclusions: Key biosynthetic enzymes of the LTB_4 and LTC_4 biosynthetic pathways are incrementally expressed across the spectrum of squamous, Barrett's metaplasia and oesophageal adenocarcinoma tissues, suggesting, for the first time, a role for both LT subfamilies in disease progression.

Keywords: arachidonate-5-lipoxygenase, Barrett oesophagus, leukotrienes, oesophageal neoplasms

Abbreviations: BM, Barrett's metaplasia; COX, cyclooxygenase; FLAP, 5-lipoxygenase-activating protein; LOX, lipoxygenase; LT, leukotriene; LTA_4H, leukotriene A_4 hydrolase; LTC_4S, leukotriene C_4 synthase; NE, neutrophil elastase; OA, oesophageal adenocarcinoma; PPI, proton pump inhibitor

Introduction

The incidence of oesophageal adenocarcinoma (OA) is rising faster than that of any other solid tumour in western countries.[1] It is associated with a poor prognosis, the 5-year survival rate being only 10–13%.[2,3] OA arises in the metaplastic columnar epithelium in the distal oesophagus, termed Barrett's metaplasia (BM). BM confers a one in 20 lifetime risk of developing OA, equating to a risk that is 30-fold to 125-fold greater than that of the general population.[4,5] BM is present in 0.5–2% of adults in the western world, and the frequency rises to 15% in those with chronic

Address for correspondence: P Boger, Department of Luminal Gastroenterology, Mailpoint CF91, Level F Centre Block, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, UK. e-mail: philboger@btinternet.com

gastro-oesophageal reflux disease.[6–8] The mechanism by which this injury triggers metaplasia and subsequent adenocarcinoma, and why this occurs in some but not all individuals, is unknown.

Exposure of the lower oesophageal mucosa to acid and bile induces injury, with the subsequent inflammatory response being an important initiating event in the progression to OA.[9] Chronic inflammation is a well-established risk factor for cancer in a number of tissue sites in the gastrointestinal tract, providing a microenvironment rich in inflammatory mediators that enhance cell growth and survival.[10–12] Among these are eicosanoids, generated upon cell stimulation by the metabolism of membrane-derived arachidonic acid by the cytochrome P450, cyclooxygenase (COX) and lipoxygenase (LOX) pathways.[13] Intracellular 5-LOX and its activating protein 5-LOX-activating protein (FLAP) metabolize arachidonic acid to an unstable intermediate, which is further metabolized by the distal enzymes leukotriene (LT)A_4 hydrolase (LTA_4H) and LTC_4 synthase (LTC_4S) to the extracellular, receptor-active LTB_4 and LTC_4, respectively.[14] LTs have diverse inflammatory actions, including leukocyte activation and chemotaxis,[15,16] prolonging neutrophil survival by preventing apoptosis,[17] and stimulating other cells to produce inflammatory cytokines.[18]

Up-regulation of the LT pathway is evident in a variety of tumour types, and plays a role in the promotion of cancer cell growth by suppressing apoptosis and promoting neo-angiogenesis.[19–21] In the oesophagus, increased levels of LTB_4 are found in BM,[22] and expression of 5-LOX and LTA_4H has been demonstrated in OA.[20,23,24] In a rat model of OA, inhibitors of 5-LOX and LTA_4H reduced tumour burden *in vivo*.[23] The full role of LTs in BM and OA and their cellular sources are nevertheless poorly defined, particularly with respect to the cysteinyl-LT subfamily. In this study, we evaluated cellular expression of the key proximal and distal 5-LOX pathway proteins (5-LOX, FLAP, LTA_4H, and LTC_4S), and related such expression to populations of pertinent cell types in oesophageal biopsies from patients with OA and its premalignant lesion, BM.

Materials and methods

SUBJECTS

Fresh and adjacent formalin-fixed oesophageal biopsies were obtained at upper gastrointestinal endoscopy from 16 subjects with BM and from seven subjects with OA. Five biopsies were taken from an area of normal squamous oesophagus proximal to the diseased segment (two from a patient with BM, and three from a patient with OA). Biopsies from those with BM were taken 2 cm proximal to the top of the gastric folds. The length of Barrett's segment and current use of proton pump inhibitors (PPIs) and aspirin were recorded. The biopsies submitted for formalin fixation were processed using routine methods into haematoxylin and eosin-stained sections, and assessed by a histopathologist expert in the field of gastrointestinal pathology, blinded to any study results. BM was defined as the visualization of metaplastic columnar epithelium with the histological finding of intestinal metaplasia.

All subjects gave written informed consent prior to participation, and the study was approved by the Isle of Wight, Portsmouth and Southeast Hampshire Research Ethics Committee (reference number: 08-H0501-3).

IMMUNOHISTOCHEMISTRY OF OESOPHAGEAL BIOPSIES

The fresh biopsy specimens were immediately fixed in ice-cold acetone containing the protease inhibitors phenylmethylsulphonylfluoride (2 mM) and iodacetamide (20 mM) (Sigma, Poole, UK) for 16–24 h at –20°C, and then embedded in glycol methacrylate resin (Park Scientific, Northampton, UK), as previously described.[25] Serial 2-μm sections were cut, floated onto ammonia water, and picked up on glass slides coated with poly (L-lysine). Immunohistochemistry was performed using the avidin–biotin complex method, as previously described.[26] Tissues were treated with a Tween-20 wash prior to application of sodium azide (0.1%) and hydrogen peroxide (0.3%) to block endogenous peroxidases. The avidin–biotin blocking kit (Vector Laboratories, Peterborough, UK) was used to block endogenous biotin, biotin receptors, and avidin-binding sites.[27]

Sections were incubated overnight at room temperature with monoclonal primary antibodies or at 4°C with polyclonal primary antibodies, as outlined below. All antibodies were used at optimized concentrations. LTC_4S antibody was diluted with 1% bovine serum albumin in Tris-buffered saline to minimize non-specific protein interactions. Secondary antibodies were biotinylated swine anti-rabbit or biotinylated rabbit anti-mouse, as appropriate (Dako, High Wycombe, UK). 3-Amino-9-ethylcarbazole (red product) was used as the chromagen when cell counts were quantified visually, and diaminobenzidine tetrahydrochloride (dark brown product) when epithelial staining was quantified by image analyser. Counterstaining was performed for 1–2 min with Mayer's haematoxylin. Negative control sections were incubated with buffer instead of the primary antibody or with non-immune rabbit serum (Sigma).

PRIMARY ANTIBODIES

Rabbit polyclonal antibody (IgG) against 5-LOX was from Cayman Chemical (Ann Arbor, MI, USA; Cat No: 160402). J. Menke (Merck & Co., West Point, PA, USA) kindly provided rabbit polyclonal antibodies against human FLAP, LTA_4H, and LTC_4S, as previously described.[28] Mouse monoclonal antibodies against neutrophil elastase (NE) (an IgG1), CD68 (IgG3κ) and CD3 (IgG1) were from Dako, and EG2 antibody against eosinophil cationic protein (IgG1) was from Diagnostics Development (Skolgatan, Sweden).

QUANTIFICATION OF IMMUNOHISTOCHEMICAL STAINING

Positive immunostaining was defined as distinct nucleated cells exhibiting staining following exposure to the chromagen. Positive cells observed near the edge of a section where a wash of stain was apparent were ignored. All other immunopositive cells were counted in each tissue section, and the average count in two non-adjacent sections was recorded. The total area of the counted tissue sections was measured in mm^2, ignoring areas of tissue disruption or folding, with a computer-assisted image analyser (Image Associates, Bicester, UK). Immunopositive cell prevalence was documented as positive cells per mm^2 of tissue. When epithelial staining was observed, image analysis software (Zeiss KS-400) was used to measure percentage epithelial area staining on the basis of red/green/colour composition, as previously described.[29]

STATISTICAL ANALYSES

Statistical tests were performed with Pasw Statistics version 18 for Windows (IBM UK, Portsmouth, UK). Unpaired Student's *t*-tests were used for comparison between groups on original or $\log_{10}$-transformed values as appropriate, depending on the data distribution as determined by normality testing. Equal variance was not assumed. Counts of cells immunopositive for each 5-LOX pathway protein were also correlated with counts of neutrophils, eosinophils, monocyte–macrophages and T lymphocytes using Pearson's correlation coefficient.

Results

IMMUNOSTAINING FOR 5-LOX PATHWAY PROTEINS IN OESOPHAGEAL BIOPSIES

Distinct nucleated cells with perinuclear immunopositive staining for each of the 5-LOX pathway proteins (5-LOX, FLAP, LTA_4H, and LTC_4S) were observed in the stromal areas of all tissue types. Representative photomicrographs from BM and OA tissue are shown in Figure 1.

There were marked differences in 5-LOX pathway protein immunostaining between the tissue types. Geometric mean cell counts (per mm^2) were significantly higher in OA tissue ($n = 7$) than in squamous tissues ($n = 5$) for 5-LOX ($P = 0.041$), FLAP ($P = 0.038$), LTA_4H ($P = 0.0008$), and LTC_4S ($P = 0.036$) (Figure 2). The increases in geometric mean values in OA tissues were approximately three-fold, eight-fold, 37-fold and six-fold, respectively, for the four proteins, as compared with squamous values. In BM tissues ($n = 16$), geometric mean counts of cells staining for 5-LOX, FLAP and LTA_4H were numerically intermediate between those of the squamous and OA tissues, although in comparison with squamous tissues the differences were not statistically significant (Figure 2A–C). In contrast, geometric counts of cells immunostaining for LTC_4S were significantly higher in both the BM ($P = 0.014$) and OA ($P = 0.036$) tissues than in squamous tissue, with no difference being seen between the two disease tissue types (Figure 2D).

Of the four 5-LOX pathway proteins, only LTC_4S showed immunostaining in both epithelial cells (Figure 1E) and stromal cells, and the epithelial LTC_4S staining appeared to be cytoplasmic more than perinuclear. The geometric mean percentage area of epithelium immunopositive for LTC_4S was elevated approximately three-fold in both the BM ($P = 0.0000003$) and OA ($P = 0.0024$) tissues, as compared with LTC_4S staining in squamous epithelium (Figure 2E).

LEUKOCYTE SUBTYPE COUNTS AND CORRELATION WITH 5-LOX PATHWAY PROTEINS

Elastase-positive (NE+) neutrophils were abundant in all tissue types (Figure 3), with geometric mean counts being seven-fold to eight-fold higher in OA tissues than in BM ($P = 0.006$) or squamous ($P = 0.11$) tissues. Eosinophils (EG2+), macrophages (CD68) and CD3 T cells all showed significant increases in the BM and OA tissues ($P < 0.05$) (Figure 3).

5-LOX and FLAP are expressed in a range of myeloid leukocytes, but expression of the distal enzymes is restricted to specific leukocyte subtypes, particularly neutrophils and macrophages for LTA_4H and eosinophils and mast cells for LTC_4S. Regression analyses showed that across all tissues ($n = 28$) the $\log_{10}$ counts of FLAP+ cells and LTA_4H+ cells

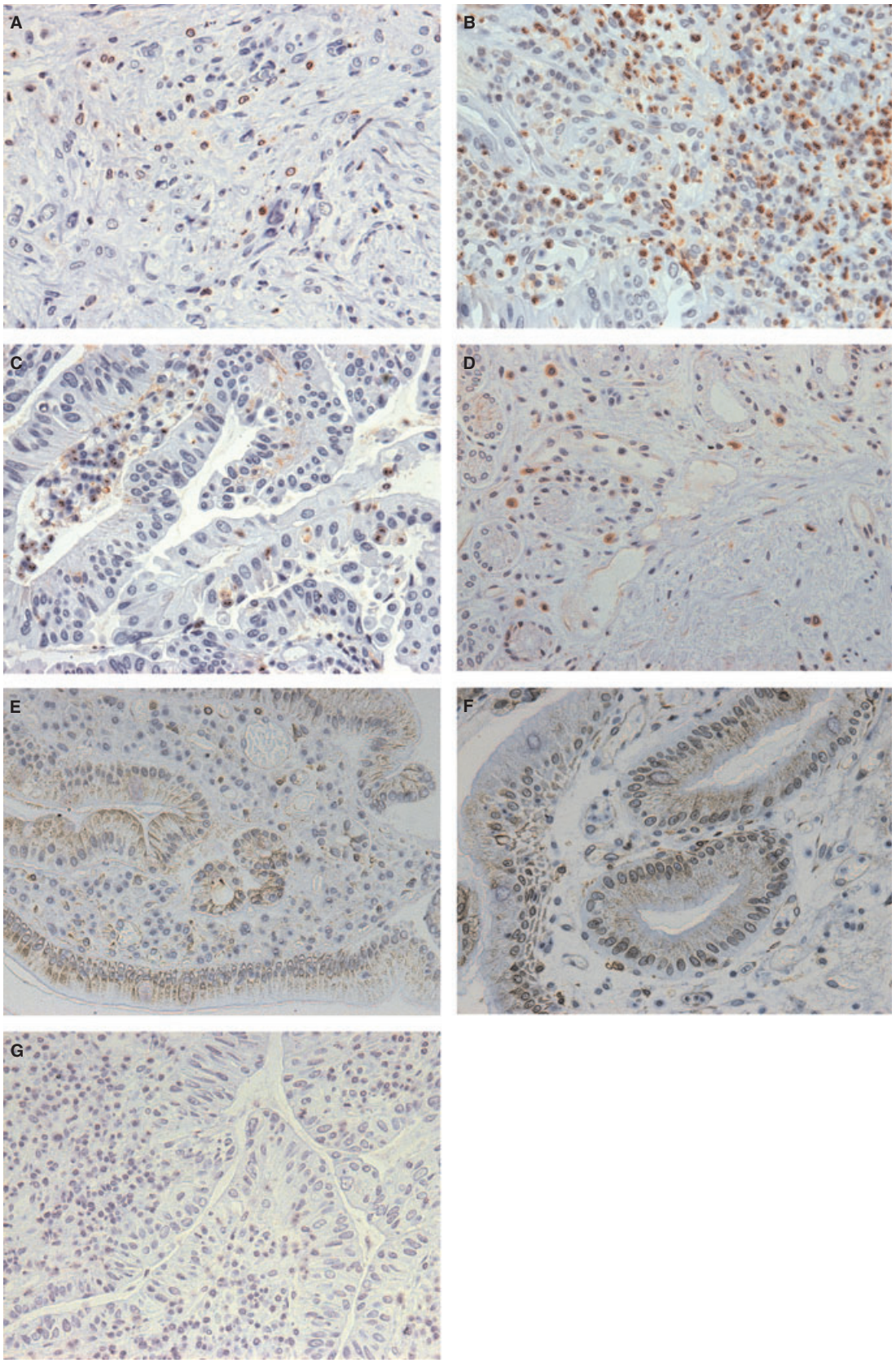

Figure 1. Immunostaining for 5-lipoxygenase (5-LOX) pathway enzymes. Representative photomicrographs show cells in the stromal area of oesophageal adenocarcinoma tissue that are immunopositive (red stain) for (**A**) 5-LOX, (**B**) 5-LOX-activating protein, (**C**) leukotriene A_4 hydrolase, and (**D**) leukotriene C_4 synthase (LTC_4S). Strong epithelial immunostaining (brown stain) for LTC_4S is shown in Barrett's metaplasia (**E**) and oesophageal adenocarcinoma tissue (**F**). **G** is the control (no primary antibody).

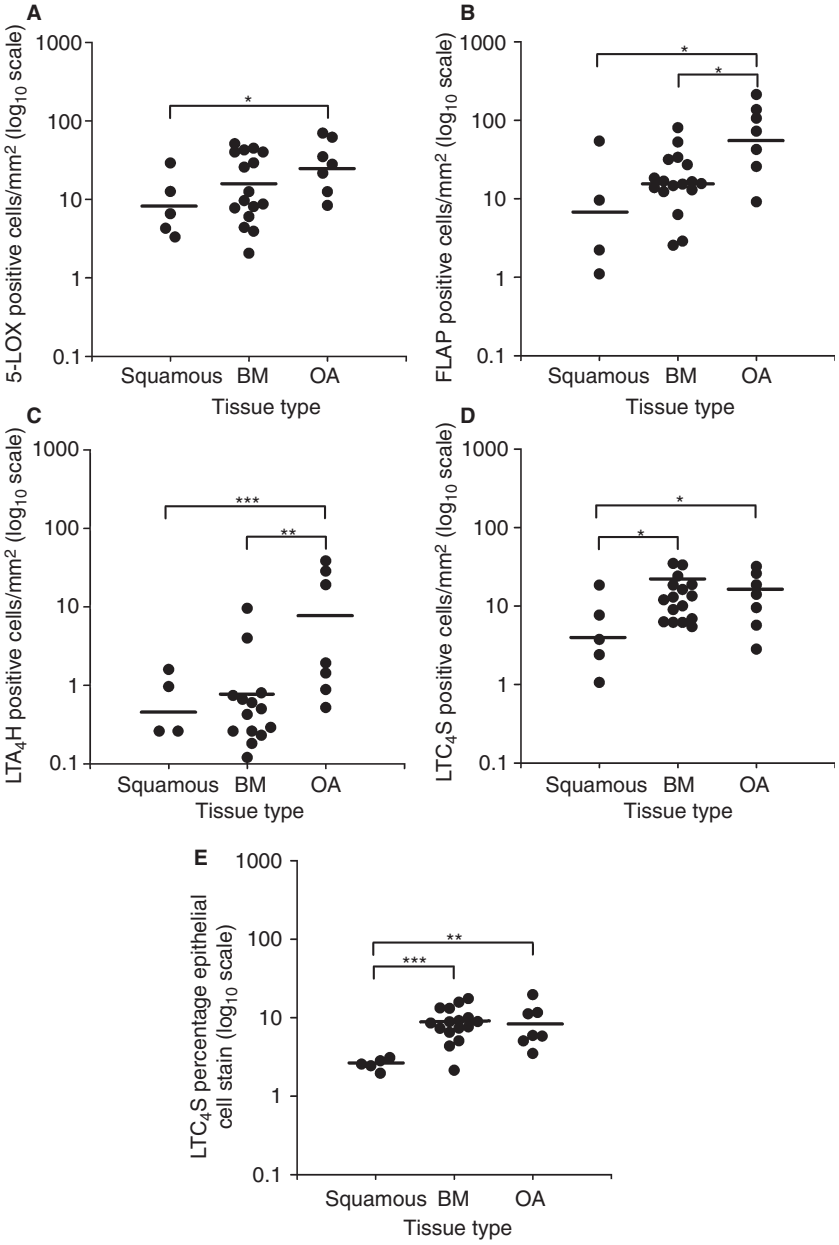

Figure 2. Expression of 5-lipoxygenase (5-LOX) pathway proteins in squamous tissue, Barrett's metaplasia (BM), and oesophageal adenocarcinoma (OA). Counts of subepithelial cells per mm^2 of stromal area that were immunopositive for (**A**) 5-LOX, (**B**) 5-LOX-activating protein, (**C**) leukotriene A_4 hydrolase and (**D**) leukotriene C_4 synthase synthase (LTC_4S) are shown ($\log_{10}$ *y*-axis) for squamous, BM and OA tissue. Epithelial LTC_4S immunostaining expressed as a percentage of epithelial area is shown in (**E**). Geometric mean values are indicated by the crosshairs, and statistical comparisons between groups were made by the use of Student's *t*-tests with $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

correlated significantly with neutrophils ($r = 0.832$ and $r = 0.736$, respectively, both $P < 0.001$) (Figure 4), whereas 5-LOX+ cell counts showed weaker correlations with CD68+ macrophages and EG2+ eosinophils ($r = 0.47$ and $r = 0.48$, respectively, both $P = 0.01$). LTC_4S counts showed no

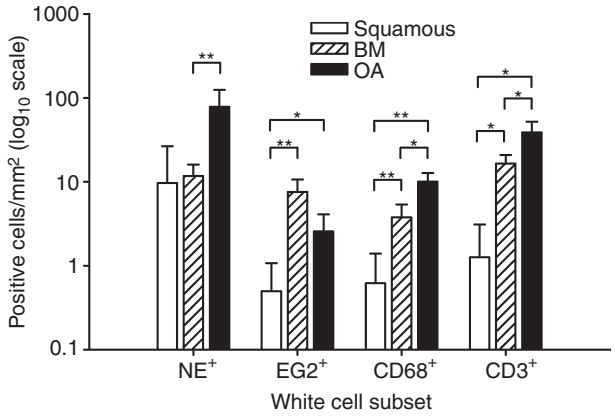

Figure 3. Leukocyte counts in squamous tissue (SQ), Barrett's metaplasia (BM), and oesophageal adenocarcinoma (OA). Counts of neutrophils immunopositive for neutrophil elastase (NE+), eosinophils immunopositive for eosinophil cationic protein (EG2+), monocyte–macrophages (CD68+) and T lymphocytes (CD3+) are shown ($\log_{10}$ *y*-axis) in SQ, BM and OA tissue. Histogram bars show geometric mean values (with standard errors), and statistical comparisons between groups were made by use of Student's *t*-tests with $^{*}P < 0.05$ and $^{**}P < 0.01$.

meaningful correlations with leukocyte counts (data not shown).

RELATIONSHIPS BETWEEN CLINICAL CHARACTERISTICS, 5-LOX PATHWAY PROTEINS AND INFLAMMATORY CELLS IN OESOPHAGEAL BIOPSIES

There were no significant differences between the groups in age or gender, and none of the patients was taking aspirin. A higher percentage of patients were taking a PPI at the time of tissue acquisition in the BM group (93.8%) than in the squamous (40%) and OA (14.3%) groups, but this difference did not reach statistical significance (chi-squared test, $P = 0.096$). To analyse the effect of concurrent PPI use at the time of tissue acquisition, oesophageal biopsy cell counts were compared between those taking a PPI ($n = 19$) and those not taking a PPI ($n = 6$). The geometric mean cell counts were numerically lower for 5-LOX, FLAP and LTA_4H and for all leukocyte subtypes (NE, EG2, CD68, and CD3) in those taking a PPI, although none of these differences reached statistical significance. The mean length of BM segment was 5.9 cm (range 3–11 cm), with no relationship to counts of cells staining positive for 5-LOX pathway proteins or leukocyte subtypes.

Discussion

There is growing recognition that eicosanoid mediators, including prostanoids and LTs, have roles not only in promoting inflammation but also in carcinogenesis, including cancers of the gastrointestinal tract. Previous studies have identified increased expression and activity of 5-LOX in patients with OA, in animal models of OA, and in OA cell lines.[20,24] With its activating protein FLAP, 5-LOX supplies an unstable substrate (LTA_4) for the synthesis of the short-lived, receptor-active lipid mediators LTB_4 and LTC_4 by the activities of LTA_4H and LTC_4S, respectively. Our study demonstrated, first, that all four of these enzymes are significantly overexpressed in OA as compared with squamous control tissue. Second, we showed that LTC_4S is also up-regulated in premalignant Barrett's tissue, with unexpectedly strong expression in the oesophageal epithelium of both disease groups. These results provide a functional basis for reports of increased LTB_4 production in OA,[22] and also point to an unsuspected role of the cysteinyl-LTs (LTC_4, LTD_4, and LTE_4) in Barrett's disease progression.

Our results confirmed that 5-LOX expression is increased in OA tissue,[24] most likely in several infiltrating leukocyte types, including macrophages and neutrophils. We also demonstrated for the first time a highly significant increase in FLAP immunoexpression in the same tissues (Figure 2). FLAP is an integral nuclear envelope protein and an essential, cytokine-inducible cofactor for LT synthesis in intact cells;[30] it is also a promising target for novel inhibitors of LT synthesis.[31] Its striking overexpression in OA tissues suggests an increased intracellular capacity to donate arachidonate to 5-LOX to initiate LT synthesis.

Subsequent generation of LTB_4 depends on cell-specific expression of the distal enzyme LTA_4H, which was markedly increased (37-fold) in OA tissues in our study, as suggested by a previous semiquantitative study.[23] Both FLAP+ and LTA_4H+ cell numbers correlated significantly with counts of neutrophils (Figure 4), which were also elevated in OA tissue (Figure 3). These results suggest that an activated 5-LOX–FLAP–LTA_4H pathway is expressed within neutrophils, and that its product LTB_4, a highly potent leukocyte chemotaxin via its BLT1 receptors,[13] is implicated in recruiting neutrophils to OA tissues. Neutrophil infiltration is observed in both acute and chronic inflammatory states, and also in different cancers throughout the gastrointestinal tract.[32] Neutrophils play an active role in the tumour microenvironment, with neutrophil-derived factors promoting genetic mutations and some, including LTB_4, mediating vascular endothelial growth factor production from tumour cells, contributing to angiogenesis.[19,33]

5-LOX pathway products are directly linked to carcinogenesis and tumour cell survival in a variety

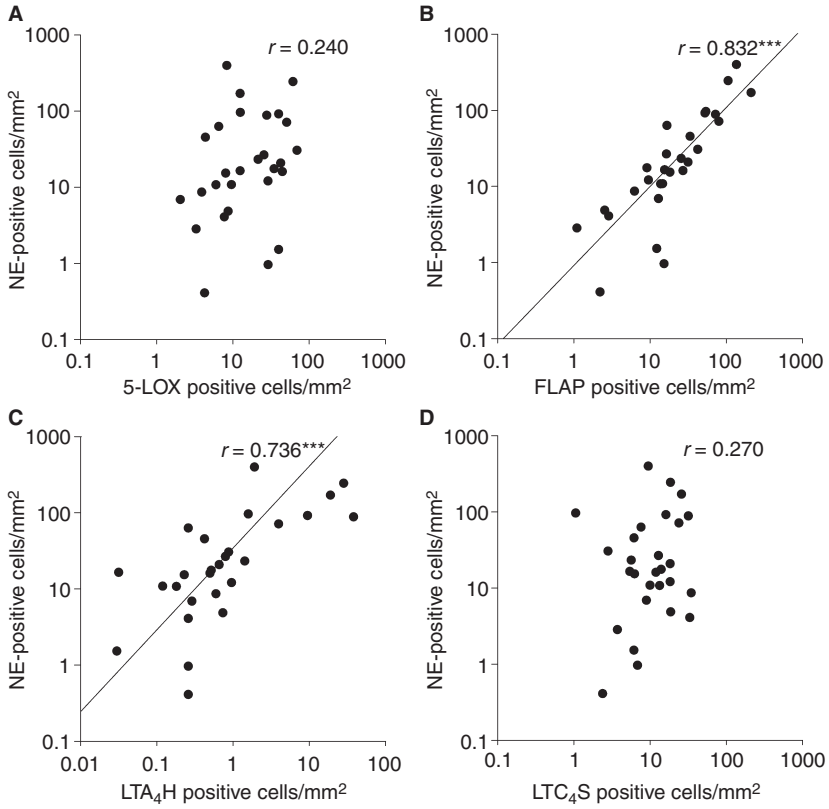

Figure 4. Correlation of 5-lipoxygenase (5-LOX) pathway protein immunoexpression with neutrophil counts. Scatter plots with line of best fit showing correlations between the counts (per mm^2) of neutrophils (neutrophil elastase-positive, *y*-axes) with counts of cells immunopositive for (**A**) 5-LOX, (**B**) 5-LOX-activating protein(FLAP), (**C**) leukotriene A_4 hydrolase (LTA_4H) and (**D**) leukotriene C_4 synthase in squamous ($n = 5$), Barrett's metaplasia ($n = 16$) and oesophageal carcinoma ($n = 7$) tissues. Pearson's correlation coefficients (r) are shown, with $^{***}P < 0.001$ for the correlations between neutrophils and FLAP and between neutrophils and LTA_4H.

of tissue sites. The marked anti-proliferative effect of LT synthesis inhibitors in breast cancer cell lines is associated with alterations in a number of apoptotic signalling proteins, including bcl-2 and bcl-2-associated X protein.[34] In rats, inhibitors of 5-LOX (zileuton) and of LTA_4H (bestatin) both reduce the incidence and tumour burden in OA,[23,24] suggesting a key role of LTB_4 in oesophageal tumorigenesis. Bestatin, however, although widely used as an adjuvant in cancer chemotherapy, has additional aminopeptidase inhibitory activity that is unrelated to inhibition of LTB_4 synthesis. Although suppression of LTB_4 production with a 5-LOX inhibitor induces apoptosis in oesophageal cancer cell lines, a selective antagonist of the LTB_4 receptor, BLT1 (U-75302), was less effective.[20] This suggests that LTB_4 receptors other than BLT1, or more probably 5-LOX products other than LTB_4, play additional roles in oesophageal cancer cell survival. Indeed, the cysteinyl-LT LTD_4 causes time-dependent and dose-dependent increases in β-catenin and bcl-2 in intestinal cell lines, and the associated reduction in cell death correlates with levels of LTD_4 as well as those of LTB_4.[35]

In this context, our novel finding of increased LTC_4S expression in OA tissue, both in stromal cells and in epithelial cells, is of particular interest. LTC_4S is an integral nuclear membrane protein and the rate-limiting step for the synthesis of LTC_4, the first of the cysteinyl-LTs, which have a range of vascular, anti-apoptotic and pro-inflammatory actions via CysLT1, CysLT2 receptors, and $P2Y_{12}$.[14,16,36,37] LTC_4S has relatively restricted cellular expression in some myeloid leukocyte subtypes, but has also been described in bronchial epithelial cells.[38] The expression of LTC_4S in oesophageal epithelial cells in our study is therefore novel, and also intriguing on two accounts. First, oesophageal epithelial cells did not express the proximal enzymes 5-LOX or FLAP, suggesting that epithelial cells may generate LTC_4 from its precursor LTA_4 donated by myeloid leukocytes in a process of transcellular synthesis, as proposed in other tissues.[39] Second, whereas 5-LOX, FLAP and LTA_4H appeared

to be more prevalent, only LTC_4S was significantly overexpressed in both BM biopsies and OA biopsies. Confidence in the finding of epithelial LTC_4S is merited by the careful measures that we took to avoid non-specific immunostaining, as described in Materials and methods. Our data suggest that stromal and epithelial LTC_4S up-regulation may represent a relatively early marker of Barrett's disease progression.

Although we did not find significant increases in the immunoexpression of 5-LOX, FLAP or LTA_4H in the BM tissues, it is possible that this may vary with the inflammatory gradient along a Barrett's segment, with inflammation being maximal at the new squamo-columnar junction and minimal distally.[40] We took biopsies 2 cm from the oesophago-gastric junction, for consistency, but, given the mean segment length of 5.9 cm in our series, this is approximately 4 cm distal from the site of maximal inflammation at the squamo-columnar junction. Further work is thus required to map the expression of the 5-LOX pathway along the entire Barrett's segment. It is also possible that alterations in enzymatic activity of the 5-LOX pathway occur without changes in enzyme expression. The findings of increased levels of LTB_4 in both oesophagitis and BM, with a significant reduction in LTB_4 levels after gastric acid production had been suppressed by a PPI,[22] suggest that gastric acid exposure may modulate LT synthesis enzymatically, perhaps by pH-dependent activation of infiltrating leukocytes. Additional risk factors, such as aspirin and other non-steroidal anti-inflammatory drugs, which inhibit COX-1, may promote LT synthesis enzymatically by the shunting of arachidonate from the COX to the 5-LOX pathway. Such effects would be magnified by the concurrent overexpression of all four 5-LOX pathway enzymes observed in OA tissues.

Overall, the translation of these laboratory findings into clinical practice will depend on understanding the complex interplay of the eicosanoid products of the 5-LOX and related pathways, such as the COX-2 products also implicated in BM and OA.[9,41–44] Dual inhibition of the 5-LOX and COX-2 pathways may be required for optimum therapy, as suggested by the additive effects of zileuton and the COX-2 inhibitor celecoxib in a rat model of OA.[24] The likelihood that different eicosanoids have pro-tumorigenic and anti-tumorigenic properties makes a better understanding of this microenvironment of paramount importance prior to extended trials of such therapy.[45]

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Conflict of interest statement

All authors state that no financial or other conflicts of interest exist.

Author contributions

P. C. Boger, P. Patel and A. P. Sampson conceived the study and experiments. P. C. Boger carried out experiments. All authors helped with data analysis. P. C. Boger and A. P. Sampson drafted the paper, and all authors gave final approval of the manuscript.

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