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Oesophageal Cancer: strategies to improve outcomes, and identify novel immunological biomarkers and targets

by

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Abstract

FACULTY OF MEDICINE

Doctor of Philosophy

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Oesophageal cancer is the most rapidly increasing cancer in the western world, the 6th most common cause of cancer death and is associated with a 5-year survival of less than 15%. This thesis aims to address current clinical issues in the management of patients with oesophageal cancer in an attempt to improve outcomes. We have assessed the impact of recent innovations in staging and minimally invasive surgery, and suggest potential novel immunological targets and biomarkers to predict response to chemotherapy, morbidity following surgery, and survival.

The impact of positron emission tomography - computed tomography in staging was observed in a multi-centre United Kingdom setting and found an additional 9% of occult distant metastases compared to traditional staging methods, justifing its use by reducing the radical treatment of patients with metastatic disease.

Minimally invasive oesophagectomy has been recently introduced into practice in a few specialist centres and we compare minimally invasive with open Ivor Lewis oesophagectomy showing there to be no detriment when compared directly for short and medium term outcomes.

With regard to improving morbidity we developed and prospectively validated a novel scoring system, based on markers of the systemic inflammatory response, to predict major complications and anastomotic leak earlier than standard postoperative care.

Both neoadjuvant chemotherapy and oesophagectomy are associated with significant morbidity. To improve outcomes further we firstly define what represents a significant immunopathological response to neoadjuvant chemotherapy, suggesting that both the response in the tumour and lymph node is adopted as a method to evaluate tumour regression, as it is these patients that have a significant benefit. We highlight immunonutritional blood-borne markers that predict both long-term survival and response to neoadjuvant chemotherapy.

In terms of novel treatments for oesophageal cancer, immunotherapy remains attractive. Current immunotherapies have not delivered significant results in solid tumours, the reasons for this being multifactorial but include the ability of the tumour to evade the immune response. We define the local tumour inflammatory environment and specific target tumour antigens, cancer testis antigens, as potential cancer vaccine targets and biomarkers in oesophageal adenocarcinoma.

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Author's declaration

I, Fergus Noble, declare that the thesis entitled Oesophageal Cancer: strategies to improve outcomes, and identify novel immunological biomarkers and targets and the work presented in it is my own. 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 and are listed in the section publications arising during candidature for thesis.

Signed: ……..

Date: ……..

Publications arising during candidature for thesis

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Underwood TJ, Hayden A, Derouet M, Garcia E, Noble F, White M, Uzoho C, Clemons N, Primrose JN, Blaydes JP, Thomas GJ. **Cancer Associated Fibroblasts Predict for Poor Outcome and Promote Cancer Cell Invasion in Esophageal Adenocarcinoma.** Journal of Pathology 2015; 235 (3):466-77.

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Abbreviations

AC	adenocarcinoma
AJCC	American Joint Committee on Cancer
AL	anastomotic leak
APC	argon plasma coagulation
APC	antigen presenting cell
ASA	American Society of Anesthesiologists
AUC	area under the curve
β-ME	β-Mercaptoethanol
BCR	B-cell receptor
BO	Barrett's oesophagus
C-D	Clavien-Dindo classification
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CF	cisplatin and 5-fluorouracil
CRM	circumferential resection margin
CRP	C-reactive protein
CSS	cancer specific survival
CT	computed tomography
CTA	cancer testis antigen
CTLs	cytotoxic T-lymphocytes
DCs	dendritic cells
DFS	disease free survival
DNA	deoxyribonucleic acid
ECX	epuribicin, cisplatin and capecitabine
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EMR	endoscopic mucosal resection
EUS	endoscopic ultrasound
FCS	fetal calf serum
FDA	Food and Drug Administration
Foxp3	forkhead box p3
Fc	fragment crystal
Fab	fragment antigen binding
FDG	[18F]-fluoro-2-deoxy-D-glucose
γδ	gammadelta
GOJ	gastrooesophageal junction
GORD	gastro-oesophageal reflux disease
GPS	Glasgow prognostic score
HER-2	human epidermal growth factor receptor 2
HGD	high grade dysplasia
HLA	human leukocyte antigen
HSPs	heat shock proteins
HTA	human tissue act
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IS	in-situ
IVL	Ivor Lewis
LGD	low grade dysplasia
LTA	left thoracoabdominal
mAb	monoclonal antibody
MAGE	melanoma antigen E
mDC	myeloid dendritic cells
MDT	multi-disciplinary team
MIO	minimally invasive oesophagectomy
MPEC	multipolar electro coagulation
NBF	neutral buffered formaldehyde
NFk-B	nuclear factor kappa-B
NK	natural killer cells
NKR	natural killer receptors
NKT	natural killer T cells
NICE	National Institute of Clinical Effectiveness
NLR	neutophil/lymphocyte index
NSAIDs	non-steroid anti-inflammatory drugs
NSTEMI	non ST elevation myocardial infarction
OAC	oesophageal adenocarcinoma
OS	overall survival
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PDS	polydioxanone
PDT	photodynamic therapy
PET	positron-emission tomography
PLR	platelet/lymphocyte ratio
PNI	prognostic nutritional index
POD	postoperative day
PSA	prostate specific antigen
PPIs	proton pump inhibitors
SCC	squamous cell carcinoma
SIM	specialized intestinal metaplasia
SRER	stepwise radical endoscopic resection
R0	complete resection
RFA	radio frequency ablation
RNA	ribonucleic acid
ROC	receiver operator curve
TAA	tumour associated antigen
TAM	tumour associated macrophages
TAP	transporter associated with antigen processing
TCR	T cell receptor
Th cell	T helper cell
TIL	tumour infiltrating lymphocytes
TNF	tumour necrosis factor
TNM	tumour, node and metastasis classification
Tregs	regulatory T cells
TRG	tumour regression grade
UHS	University Hospital Southampton
UK	United Kingdom
UoS	University of Southampton
USA	United States of America
w/v	weight volume percentage
WCC	white cell count

1. Chapter 1: Introduction

1.1. Aims

The research is split in two parts firstly assessing recent innovations that serve to improve on perioperative care either by improved staging, surgical technique or postoperative care. The second part of the thesis offers potential strategies to provide targets for immunotherapy and biomarkers of prognosis, progression and response to neoadjuvant chemotherapy.

The first part of the thesis has three aims: (A) To evaluate two recent innovations in the care of patients with oesophageal cancer: firstly the use of PET/CT, aiming to improve preoperative staging described in chapter 3, and secondly (B) to compare two stage minimally invasive versus Ivor Lewis oesophagectomy, its open counterpart, in chapter 4. (C) To address the significant morbidity and mortality experienced after oesophagectomy we develop a novel risk score to predict complications early using markers of the systemic inflammatory response in chapter 5.

The second part of the thesis serves to evaluate the co-expression of immunological markers in a representative modern series of radically treated oesophageal cancer patients with a further four aims. This was with the aim of defining targets for immunotherapy and biomarkers of prognosis, progression and response to neoadjuvant chemotherapy. (D – and others below) Prior to assessing markers of response to chemotherapy, we sought to assess what constitutes a response to chemotherapy in chapter 6. (E) We initially assessed the systemic inflammatory response and its association with nutritional status in chapter 7 before going onto to evaluate (F) the local immune infiltration in chapter 8.

In terms of novel treatments for oesophageal cancer, immunotherapy remains attractive. (G) We have therefore evaluated the expression pattern of CTAs both as potential biomarkers as well as targets for immunotherapy by going on to assess the immunogenicity of a select group of CTAs in chapter 9.

1.2. Overview

Oesophageal cancer is a significant and increasing health problem. In 2005, there were 497,700 new cases globally and the prevalence is expected to increase by approximately 140% by 2025 (1). Moreover, cancer of the oesophagus and gastrooesophageal junction (GOJ) is the most rapidly increasing cancer in the western world, now the 6th most common cause of cancer death and is associated with a 5-year survival of less than 15% (2). This poor prognosis is due to the majority of patients presenting late with metastatic disease or being medically unfit, with only one in five patients deemed suitable for radical and potentially curative treatment.

In the United Kingdom (UK), oesophagogastrectomy is performed as the primary treatment for local and loco-regional disease with or without neoadjuvant chemotherapy

depending on the stage of the tumour. Both interventions are associated with high morbidity and mortality (3) and so improvements in diagnosis, treatment and staging are required. Prognostic markers that can predict poor prognosis or response to chemotherapy would be of additional benefit in making treatment decisions.

This introduction aims to provide relevant background to oesophageal cancer focusing on radical and potentially curative treatments, current biomarker research, tumour immunology and summarising current immunological markers in oesophageal cancer.

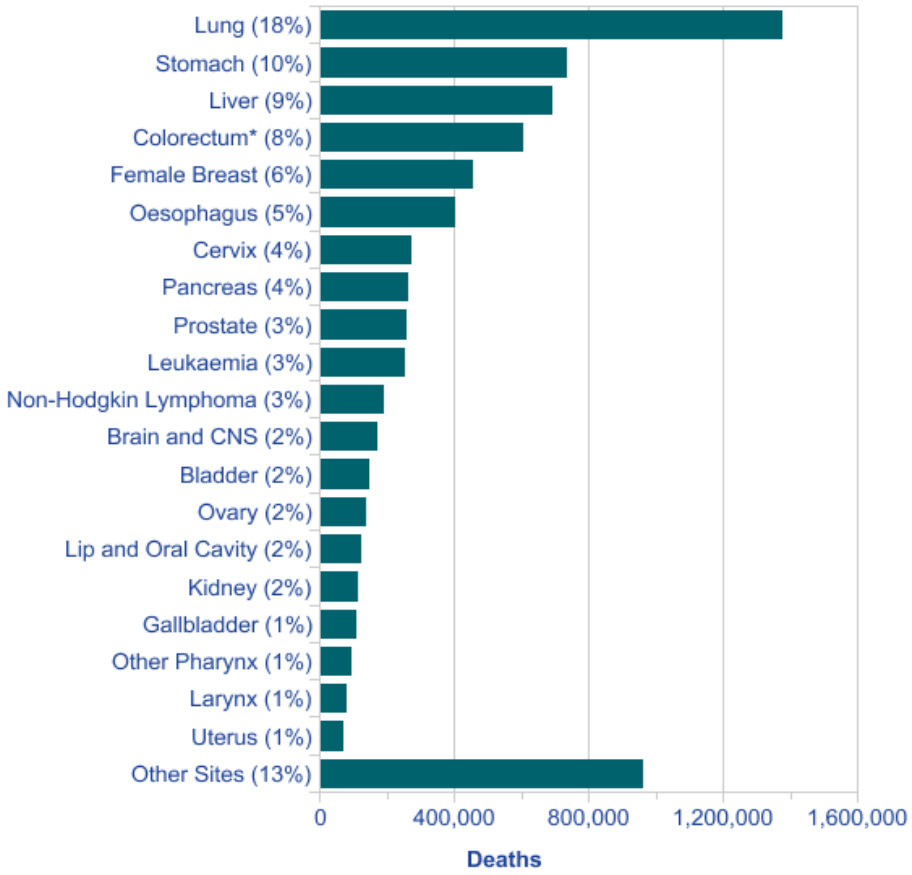


Figure 1-1: The 20 most common causes of death from cancer worldwide, 2008 estimates (Cancer research UK, 2011)

1.3. Oesophagus

1.3.1. Anatomy of the oesophagus

The oesophagus is a muscular tube occupying the posterior mediastinum connecting the cricopharyngeal sphincter to the cardia of the stomach and is approximately 25cm long in the adult (4).

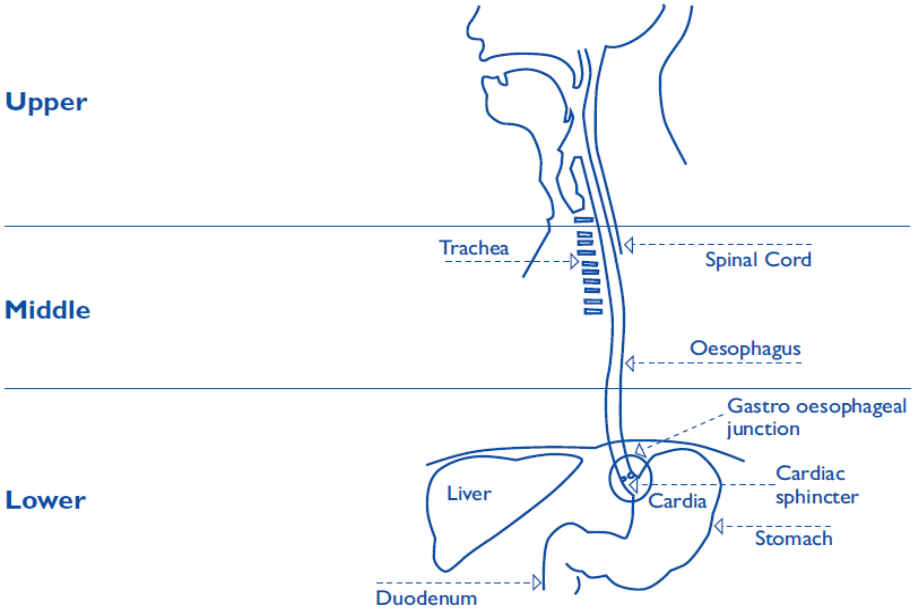


Figure 1-2: Diagram of the oesophagus (Cancer research UK, 2011)

1.3.2. Physiology of the oesophagus

The oesophagus can be divided into three separate sections: cervical, thoracic, and lower oesophagus. Within these sections are narrowings that include two high pressure areas that remain relatively contracted in the resting phase, the lower and upper oesophageal sphincters (5). These help in carrying out its main function of transporting food to the cardia of the stomach via peristalsis whilst avoiding reflux of the gastric contents back into the oesophagus. The upper oesophagus is made up of striated muscle, helping propulsion, whereas the lower oesophagus is mainly composed of smooth muscle under autonomic neural control (6, 7).

Control seems to rely on four factors: diaphragmatic contraction, greater intra-abdominal pressure than intra-gastric pressure being exerted upon the abdominal part of the oesophagus, unidirectional peristalsis and maintenance of correct anatomical arrangements of the structure (8, 9). This tight control prevents reflux of gastric contents back into the oesophagus. Factors such as obesity, hiatus hernia and drugs that reduce this control are implicated in the increase in reflux and oesophageal adenocarcinoma (OAC) (10-12).

1.1.3. Histology of the oesophagus

The oesophagus consists of a mucosa, submucosa, muscalaris propria and adventitia similar to the rest of the gastrointestinal tract (13).

The mucosa is made up of a non-keratinizing squamous epithelial layer that overlies the lamina propria. It is this basal layer that proliferates to replace the cells that are lost. Squamous cell carcinoma (SCC) is derived from this layer (14). The lamina propria is the non-epithelial part of the mucosa consisting of immune cells, connective

tissue, vascular structures and oesophageal cardiac glands, which are mucus secreting (5). It is from these glands that adenocarcinoma (AC) arises (6).

The submucosa consists of loose connective tissue supporting the mucosa containing the blood vessels and lymphatic networks.

The oesophagus is covered by fibrous adventitia along its length which blends with surrounding tissue rather than serosa like in the majority of the gastrointestinal tract. This absence of serosa, in addition to a dense lymphatic network, facilitates the spread of infections and metastasis from the oesophagus into the posterior mediastinum (5).

As the oesophagus is normally lined with squamous cells, SCC can occur anywhere in the oesophagus. There is increasing but not conclusive evidence that OAC can only develop if squamous epithelium is replaced by metaplastic columnar epithelium and so usually occurs in the lower oesophagus or GOJ (15, 16). This occurs predominately in patients with gastro-oesophageal reflux and is known as Barrett's oesophagus (BO) and was first described in 1950 (17).

1.4. Oesophageal cancer

1.4.1. Epidemiology

1.4.1.1. Squamous cell carcinoma

The incidence varies considerably with respect to geographical location and to a lesser extent by social and ethnic groups. Worldwide the commonest tumour of the oesophagus remains SCC in contrast to Western Europe and the United States of America (USA). The highest rates occur in northern China and northern Iran where incidence is more than 100 per 100,000 of the population. In the USA, SCC rates fell by 3.6% each year between 1998 and 2002 and this occurred in most ethnic groups and in both sexes (18). This decrease is thought to be due to a reduction in smoking (19).

1.4.1.2. Adenocarcinoma

In the Far East, no increase in AC has been observed, and SCC continues to be the more common cancer (20).

In the UK, the majority of patients with oesophageal cancer in the past were diagnosed with SCC of the thoracic oesophagus whereas now the most common type in the UK is AC of the lower oesophagus and GOJ with age-standardised incidence rates of AC rising by just under 40% every 5 years (21, 22). The UK has the highest incidence of OAC globally, at 7 cases per 100,000 of the population (22, 23). The majority of cases occur in caucasian males, but other ethnic groups also develop AC of the oesophagus (1).

1.4.2. Aetiology

Most patients are diagnosed between 55 and 85 years old and are male. The aetiology is multifactorial, and the striking variations in the incidence rates over time and between populations is not understood but is believed to be caused by differences in environmental risk factors and rising obesity (23-25).

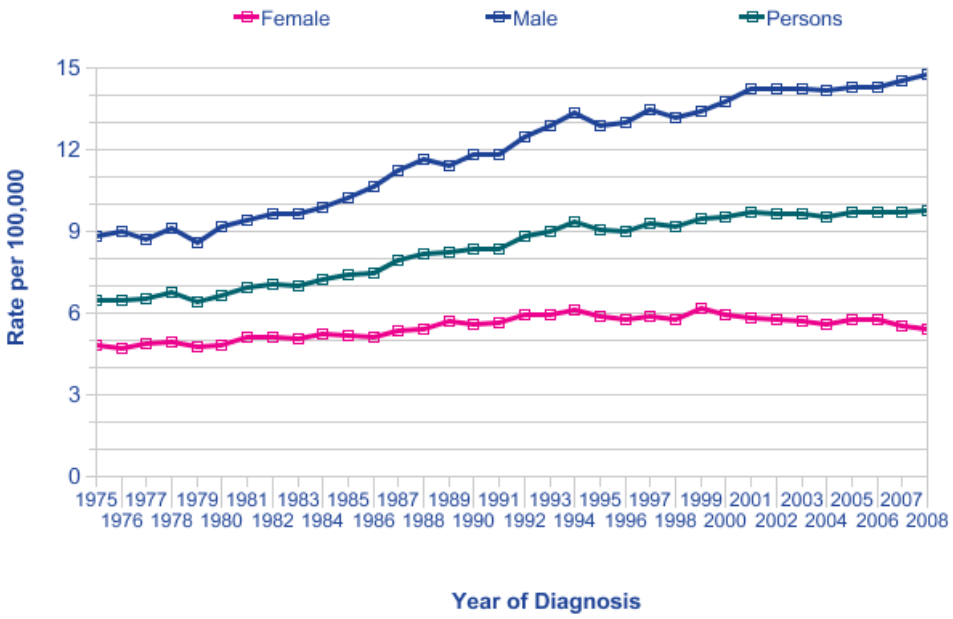


Figure 1-3: Oesophageal cancer, European age-standardised incidence rates, Great Britian, 1975-2008 (Cancer research UK, 2011)

1.4.2.1. Risk factors

1.4.2.1.1. Squamous cell carcinoma

In Linxian, HuMan Province China, oesophageal SCC is endemic and has been directly related to nitrosamines and inversely related to consumption of riboflavin, nicotinic acid, magnesium and zinc (26).

In the western world there is less impact from dietary factors due to differences in food preservation techniques and the primary cause is the use of alcohol and tobacco, which have a synergistic effect (27). The risk is directly associated with the number of cigarettes smoked per day and the duration of smoking (28-30). This may explain the higher incidence in men.

Further established risk factors include socioeconomic deprivation and low consumption of fruit and vegetables (31-33). Other risk factors include achalasia, Plummer Vinson syndrome and frequent consumption of hot beverages that all lead to chronic inflammation (34, 35).

Tylosis a rare autosomal dominant disorder defined by a genetic abnormality at chromosome 17q25, is the only recognized familial syndrome that predisposes patients to SCC of the oesophagus (36). It leads to a 95% risk of SCC by 70 years of age (37).

1.4.2.1.2. Adenocarcinoma

The greatest association for OAC is found with gastro-oesophageal reflux disease (GORD) (38). Other risk factors include obesity, hiatus hernia, tobacco smoking and certain medications, all of which may act through predisposing to gastro-oesophageal reflux (39).

In addition, diets high in fat, calories and cholesterol but low in antioxidants and fibre confer an increased risk (40).

In contrast helicobacter pylori infection has been demonstrated to offer protection against the development of BO (41) and OAC (42, 43). This is thought to be due to a reduction in reflux and acid (44). There are conflicting studies regarding whether non-steroid anti-inflammatory drugs (NSAIDs) decrease the risk of developing OAC. The AspECT trial is currently looking at the use of aspirin and proton pump inhibitor's (PPIs) in BO patients in the UK (45, 46).

1.4.2.1.3. Barrett's oesophagus

Gastro-oesophageal reflux is common in the western world with as many as 30% of the population experiencing monthly symptoms and approximately 10% of gastro-oesophageal reflux patients eventually developing BO (39). However, it is unclear at a molecular level how gastro-oesophageal reflux leads to oesophageal carcinogenesis in BO patients. Prolonged acid and bile reflux are common in BO patients (47, 48).

OAC is thought to develop from BO, where the normal squamous epithelium is replaced by a metaplastic columnar type epithelium. Although several other types of columnar epithelium can be found in the lower oesophagus it is suggested that only specialised intestinal metaplasia (SIM), distinguished by the presence of goblet cells, confers an increased cancer risk. It is likely the triggers for progression are multifactorial in nature, involving chromosomal aberration, genetic and epigenetic events, as well as environmental factors. The likely process is for metaplastic cells in patients with BO to accumulate genetic alterations and progress through dysplasia to OAC over many years as these abnormal cells are selected to proliferate with a survival advantage over normal cells in a process termed clonal expansion (49, 50).

The published literature indicates that 19–59% of cases of high-grade dysplasia (HGD) progress to cancer (51). Thus oesophagectomy has been suggested for those fit for surgery providing disease free survival (DFS) of 100% at 32 months in a recent series. It is also noted that occult adenocarcinoma was found in 29% of cases of HGD. This is comparable to available studies dating back to 1983 revealing a 37% incidence of occult carcinoma in HGD (52).

There are numerous endoscopic ablative techniques now available photodynamic therapy (PDT), laser, multipolar electro coagulation (MPEC), argon plasma coagulation (APC), radiofrequency ablation, cryotherapy, as well as endoscopic mucosal resection (EMR). PDT is the only ablative technique with Food and Drug Administration (FDA) approval for HGD of the oesophagus. As well as the risk of occult AC all ablative techniques and EMR have the potential to leave behind submucosal SIM. This is reported after PDT in up to 51.5% (53). The significance is not yet determined but AC arising under squamous re-epithelialisation has been reported in up to 4.6% of cases (54, 55). The latest ablative technique to rise to prominence is radiofrequency ablation (RFA) with the most recent clinical trial assessing RFA versus a sham endoscopic procedure. This demonstrated disease progression and eradication of dysplasia improved with the ablative technique at 12 months of follow-up. Interestingly approximately 20% of patients with low grade dysplasia (LGD) and HGD had no evidence of dysplasia at 12 months with the sham procedure. This is likely to reflect the difficulty in gaining a representative biopsy. RFA has also been compared to stepwise radical endoscopic resection (SRER) both providing complete removal of intestinal metaplasia in 95% and 96% of cases respectively. However, SRER required a median of six sessions leading to dilatation for strictures in 86% of cases (56).

Whether ablative techniques eliminate the risk of cancer, the need for surveillance and are cost effective remains to be proven. It would seem sensible for ablative and mucosal resection techniques to be compared to modern series of oesophagectomy for HGD or T1 cancer rather than comparison's to the morbidity and mortality of historical series covering all stages of disease.

However ablative and mucosal resection techniques may have advantages over conventional treatments and are attractive due to the presumed reduced morbidity when compared to surgery. The risks of stricture and haemorrhage are not insignificant with ablative techniques and its use in conditions that confer minimal chance of progression to cancer is still debatable. The risk of under staging oesophageal cancer and potential risk of occult AC in patients receiving ablative and mucosal resection techniques requires close surveillance and assessment. The national institute of clinical effectiveness (NICE) recently reached a consensus over the use of RFA in the UK concluding adequate evidence for its use in HGD in addition to long-term follow-up.

There is currently no suggested medical treatment of BO other than the symptomatic treatment of GORD. Although the recent guidance from NICE regarding RFA for low grade dysplasia or BO is far from clear. Surgical treatment of BO with anti-reflux surgery is not currently recommended. It is currently used in the management of patient's whose GORD symptoms do not respond to medical therapy. The incidence of OAC in BO is low and so it is difficult to assess significant benefits of surgery over medical

therapy. A large meta-analysis did not see any difference in risk of progression between those that had anti-reflux surgery and those on medical therapy (57).

However HGD is treated, the challenge remains in predicting which patients with BO are likely to progress to cancer. The currently used biomarker of progression, grade of dysplasia, is not ideal. Problems include reliability, often a lack of inter-observer agreement, incomplete sampling and the requirement for an invasive procedure. So the search remains for better predictors of progression to OAC to provide improved risk stratification, preventative treatments, screening and surveillance regimes (58).

1.4.3. Pathology

1.4.3.1. Histological type

The commonest tumour type in the UK is AC. Other types include SCC, adenosquamous and small cell carcinomas. In general there is no difference between prognosis between the two commonest tumour types although it is suggested AC has improved prognosis over SCC for early lesions (59, 60).

1.4.3.2. Pathological features

In patients radically treated, pathological nodal stage (pN) has been shown to be the most significant prognostic indicator of survival (59, 61-64). depth of invasion (pT stage) (59, 61, 63, 65, 66)and distant metastasis (pM stage) (67) have also been shown consistently to be independently associated with overall survival (OS) and DFS and form the remainder of the TNM classification (68).

Increased size of tumour (69, 70) and polypoid type (71) have been associated with poor survival. Other features that have been reported to have survival benefit are lack of perineural invasion (72), vascular invasion (63, 64, 73), lymphatic invasion (74, 75) and completeness of surgical resection (R0) (76, 77). Whilst it is accepted that the involvement of proximal margin (62, 63, 78, 79) confers worse survival, there is greater uncertainty for distal resection margin (63, 80) and circumferential resection margin (CRM) (76, 77).

The presence of Barrett's metaplasia in the pathological specimen has been shown to be associated with improved prognosis (81). This may reflect earlier tumours as patients may have been involved in surveillance programs or it reflects tumour overgrowth of Barrett's metaplasia in more advanced tumours (16).

The significance of differentiation and survival is less clear with conflicting reports (59, 61, 62). This may reflect the heterogeneous nature of tumours.

The pathological tumour stage is determined using the American Joint Committee on Cancer (AJCC) staging system for oesophageal carcinoma. A tumour of the oesophagus or whose epicenter is within 5 cm of the GOJ and also extends into the oesophagus is classified and staged according to the oesophageal scheme (Table 1-1).

All other tumours with an epicenter in the stomach greater than 5 cm from the GOJ or those within 5 cm of the GOJ without extension into the oesophagus are staged using the gastric carcinoma scheme (68).

Despite extensive pathological prognostic markers, some patients with favourable markers have poor outcome and the identification of alternative prognostic markers would be beneficial. Preoperative prognostic markers and predictors of progression and response to treatment would potentially be of most benefit.

Table 1-1: AJCC Staging System for Oesophageal Carcinoma

Primary tumour (T):

TX:	Primary tumour cannot be assessed
T0:	No evidence of primary tumour
Tis:	Carcinoma in situ /high-grade dysplasia
T1:	into lamina propria or submucosa
T1a:	into lamina propria or muscularis mucosae
T1b:	into submucosa
T2:	into muscularis propria
T3:	into adventitia
T4:	into adjacent structures
T4a:	into pleura, pericardium, diaphragm, or adjacent peritoneum
T4b:	into other adjacent structures e.g. aorta, vertebral body, trachea

Regional lymph nodes (N):

NX:	Regional lymph nodes cannot be assessed
N0:	No regional lymph node metastasis
N1:	1 to 2 regional lymph nodes
N2:	3 to 6 regional lymph nodes
N3:	>6 regional lymph nodes

Distant metastasis (M):

MX:	Distant metastasis cannot be assessed
M0:	No distant metastasis
M1:	Distant metastasis

AJCC stage groupings

Stage IA	T1N0M0
Stage IB	T2N0M0
Stage IIA	T3N0M0
Stage IIB	T1-T2N1M0
Stage IIIA	T4aN0M0; T3N1M0; T1-2N2M0
Stage IIIB	T3N2M0
Stage IIIC	T4aN1-2M0; T4bN0-3M0; TX-4N3M0
Stage IV	TX-4NX-3M1

1.4.3.3. Tumour regression secondary to neoadjuvant therapy

The pathological response to chemotherapy is most widely assessed using Tumour Regression Grading (82) (TRG) as described by *Mandard et al (83)* although this has not gained universal acceptance. This system is based on the amount of residual tumour and the degree of fibrosis at the primary tumour (83). Other proposed pathological systems for measuring neoadjuvant treatment response include complete pathological response (84), size of residual tumour (85), number of residual tumour cells (86, 87), response classification system (88), size based pathological response (85) and downstaging of cT and cN stage (89). These grading systems have predominately been developed following chemoradiotherapy with heterogeneous histology with few studies assessing their utility following chemotherapy in patients with oesophageal adenocarcinoma (90-93). A number of clinically important questions could be addressed by a robust and universally accepted measure of response to neoadjuvant treatment including: the ability to accurately predict an individual patient's tumour response (via molecular biomarkers or imaging) to preoperative therapy leading to non-responders proceeding directly to surgery or being considered for alternative neoadjuvant regimes; assessment of new neoadjuvant regimes, and identification of patients who are likely to benefit from adjuvant therapy.

Neoadjuvant chemotherapy is currently suggested for those patients radically treated, based on improved survival (3, 94). However not all patients respond to neoadjuvant therapy and these patients have surgery delayed without benefit. Predictive markers of response would therefore be desirable to individualise patient treatment to those likely to respond to therapy (95).

We have therefore assessed pathological response to neoadjuvant chemotherapy by assessing the tumour response as well as the response in the lymph nodes in a large contemporary cohort of patients with oesophagogastric adenocarcinoma managed with neoadjuvant platinum based triplet chemotherapy, and describe their associations with short- and long-term outcomes in chapter 6.

Table 1-2: Tumour regression scoring according to Mandard et al (83)

Grade	Definition
TRG 1	No residual cancer
TRG 2	Rare residual cancer cells
TRG 3	Fibrosis outgrowing residual cancer
TRG 4	Residual cancer outgrowing fibrosis
TRG 5	Absence of regressive changes

1.4.4. Diagnosis

Oesophageal cancer rarely causes early symptoms and at the time of diagnosis more than 50% of patients have either unresectable tumours or radiologically visible metastases (96).

The majority of patients with oesophageal cancer have dysphagia (74%) and 17% report odynophagia at the time of diagnosis (97). Weight loss is also common and occurs in up to 70% and is an independent indicator of poor prognosis if 10% of body mass is lost (98). Dyspnoea, cough, hoarseness and pain (retrosternal, back or abdominal) occur less often but may indicate the presence of advanced disease (99). GORD symptoms occur in approximately 24% of patients (100) but there is poor sensitivity to OAC as similar symptoms occur in the wider population, without OAC, in up to 30% people (39).

Physical examination may reveal signs of secondary disease such as lymphadenopathy, hepatomegaly, pleural effusion or cutaneous metatasis.

Histological diagnosis is confirmed by biopsy at the time of endoscopy.

1.4.5. Staging

Conventional imaging used for staging oesophageal cancer in the UK includes contrast-enhanced computed tomography (CT) of the chest and abdomen (+/- neck) with positive or negative oral contrast medium load and endoscopic ultrasound (EUS) techniques that provide high-quality anatomical information. EUS enables accurate assessment of the depth of invasion (T stage) together with accurate information regarding local nodal involvement (N stage).

Recently preoperative imaging has variably included the use of integrated positron-emission tomography and CT (PET/CT). PET detects metabolically active tissue based on the metabolism of glucose (101). PET/CT combines both multisection CT and PET capabilities in two sequential scans, avoiding the need for patient motion between the CT and PET components of the study, thereby leading to more accurate co-registration of the CT and PET data (102).

Postoperative quality of life is significantly compromised for 3-6 months and never returns in those who develop early postoperative recurrence or metastatic disease (103). Poor long-term survival for patients who appear to have complete tumour resection appears to be, in part, due to a failure to detect distant metastases at or before the time of surgery (104). Accurate preoperative staging is thus essential in providing informed treatment choices for these patients and we assess the impact of PET/CT in chapter 3.

1.4.6. Treatment of oesophageal cancer

For fit patients presenting with operable oesophageal cancer the current standard of care in the UK is neoadjuvant chemotherapy: 2 cycles of cisplatin and 5-fluorouracil (CF), followed by resection. This treatment pathway follows the optimum arm of the Medical Research Council OE02 study (94). The expected 2 year survival rate from this approach is 43% compared with 34% for surgery alone. The current national study, OE05, commenced in 2006 and addresses whether intensification of neoadjuvant chemotherapy with epirubicin, cisplatin and capecitabine (ECX) confers a survival advantage over the standard treatment. Oesophagectomy is generally recognized as

offering the best prospect of long-term cure but is a major procedure with perioperative mortality in modern series of 0-10% and significant morbidity in up to 60% of patients (105).

To significantly improve on the overall 7-15% 5 year survival of patients presenting with oesophageal cancer a multi-faceted approach is required. Such strategies will include prevention, identification and surveillance of at risk groups, early diagnosis and improved treatments.

1.4.6.1. Screening and surveillance

BO is associated with a risk of progression to cancer of about 0.12-1% per year (106-108). Endoscopic surveillance with random biopsy sampling is therefore recommended for patients with BO (109, 110). The risk of developing OAC in non-dysplastic BO is relatively low and the cost-effectiveness of routine surveillance in low risk Barrett's patients has been questioned (106).

Due to the poor sensitivity of GORD symptoms in predicting a diagnosis of BO, endoscopic screening of patients with GORD is not recommended by British or American guidelines.

In routine clinical practice BO is not treated either by ablative techniques or by anti-reflux surgery but followed up with endoscopy over long time periods (109-111). There is however some support for the principle of screening high risk patient groups (110, 112). The call for biomarkers that predict progression to OAC is therefore warranted.

1.4.6.2. Surgical treatment

Oesophageal cancer without distant metastases in the UK is most commonly resected using the right transthoracic approach (113). This approach combines a laparotomy and right-sided thoracotomy, leading to oesophagogastric anastomosis in the upper chest (Ivor Lewis (IVL) technique) (114). Advocates suggest that a combined trans-abdominal and trans-thoracic approach offers the optimum strategy for tumour clearance and two-field lymphadenectomy (115-117). In addition the gastric resection required for adequate clearance of junctional tumours may reduce the length of the gastric conduit which would otherwise be required to reach the neck and potentially compromise the anastomosis (118, 119). This is reflected in the 2010 UK National Oesophago-Gastic Cancer audit, where nearly three quarters of operations were performed by the IVL approach (120).

Other techniques include: Mckeown (121), with a right transthoracic approach with the addition of dissection and anastomosis in the neck; the transhiatal (122) approach, involving a laparotomy with blunt dissection of the thoracic oesophagus placing the anastomosis in the neck and finally the left thoracoabdominal oesophagectomy, with enthusiasts noting excellent exposure due to simultaneous abdominal and chest exposure

but reportedly giving rise to increased post-operative pain and subsequent morbidity (123). Neither retrospective nor prospective trials have demonstrated any statistically significant differences in survival or operative mortality between transhiatal and transthoracic approachs (124-127). Subgroup analysis in one randomised control trial has shown that the transthoracic approach conferred a survival advantage for siewert type 1 tumours of the GOJ and node positive tumours.

Despite the enthusiasm for a two stage approach in the open setting, the uptake of minimally invasive two stage oesophagectomy (MIO-2) has been slow (128). The goal of minimally invasive surgical techniques is to reproduce the open operation in a format that reduces surgical trauma and improve patient outcomes. This is especially pertinent to oesophageal cancer operations where surgery in two body cavities is associated with significant morbidity. The first hybrid MIO was reported by Cuschieri nearly twenty years ago (129). Initial experiences raised concerns over safety with respiratory complications reported in as many as 59% of cases (130). Minimally invasive skills and equipment have subsequently advanced and MIO has re-emerged in a variety of forms. Recent systematic reviews and meta-analyses have concluded that MIO may reduce morbidity, hospital stay and is at least comparable to open resection for perioperative outcomes (115, 128). More recently MIO has been associated with a rapid restoration of health-related quality of life (131). Up to 30% of oesophageal resections in the UK are presently conducted using minimally invasive techniques, but only a small proportion have reported thoracoscopic oesophageal mobilisation (120). The majority of published series report the outcomes of hybrid type or three stage MIO (115, 117, 128, 132-140), with few series of MIO-2 in the literature (115, 118, 119, 141-146) and no series that directly compare MIO-2 with IVL, the standard procedure against which all other techniques are measured (147). The true benefit of MIO has yet to be defined and we explore its impact in chapter 4.

Multi-institutional randomized trials looking at surgery as a whole have documented resectability rates of 54-69%, operative mortality of 0-10%, and rates of perioperative complications of 26-41%. Patients undergoing surgery as the sole treatment had median survival ranging from 13 to 19 months, 2 year survival rates ranging from 35-42% and 5 year survival rates from 15-24% (94, 148, 149).

1.4.6.2.1. Predictors of morbidity and mortality

Morbidity and mortality occur in significant numbers following oesophageal resection despite improvements in preoperative staging(150), patient selection (151), surgery (152) and perioperative care (153). Major complications including anastomotic leak (AL) are reported at rates of 3.8-15% (154, 155) with mortality rates varying between 0-11% in modern series (156, 157). The majority of patients who develop an AL or a major complication following oesophagogastrectomy are diagnosed when symptomatic or critically ill (158) and often require intensive care management and reoperation. Rapid diagnosis of AL is essential to limit contamination and minimise sepsis (158) as this may

lead to improved clinical outcomes both in the short and long term. There is gathering evidence that septic complications, including AL, lead to reduced disease free and cancer specific survival (CSS) in colorectal cancer (159) and initial reports show this may also be the case in oesophageal and gastric cancer (160-163). Furthermore, patients assessed as being at low risk of developing a major complication may also gain advantage by being more confidently fast-tracked to enteral feeding and earlier discharge (164).

1.4.6.2.2. Preoperative

Preoperative markers have been extensively investigated for patients who undergo oesophageal surgery. They provide important information for appropriate selection of patients for oesophagectomy (105) and allow an informed decision by the surgeon and patient. In addition they will become increasingly useful, as institutions and surgeons are compared, by enabling risk adjustment for their case mix (165). Clinical characteristics reported to have associations with short term patient outcome include age (166), presence of comorbidity (105, 167, 168) and hospital volume (169). More controversy exists about the relationship of surgical approach, neoadjuvant therapy and preoperative bleeding volume with short term outcome (170). Few preoperative markers that predict outcome can be modified but this does not detract from their usefulness in clinical decision making. In addition, the knowledge of a patient's potential risk of complication can change the management of a patient.

1.4.6.2.3. Intraoperative

Intraoperative variables reported to have associations with postoperative outcome include, intraoperative blood loss, duration of operation, the need for blood and fluid replacement during and after surgery, cardiorespiratory instability, one lung ventilation and the need for inotropic support during surgery (168, 171-173). The identification of intraoperative risk factors is of use to the surgical team as they can, potentially, modify some of these risks preoperatively or change management intra- and postoperatively.

1.4.6.2.4. Postoperative

Postoperative predictors of morbidity and mortality following oesophageal surgery are scarce with the majority predicting prognosis after the complication has occurred or focus on mortality. Predictors include serum albumin, organ dysfunction and requirement for mechanical ventilation (156). Postoperative biochemical markers of the systemic inflammatory response are measured routinely following oesophagogastric resection despite limited objective evidence for their benefit. To date, only a handful of studies have assessed their association with postoperative complications. One study found low albumin on the first post operative day to be associated with major complications but not AL (174). Other studies have largely focused on C-reactive protein (CRP) following colorectal surgery (175-178), with only two looking at oesophageal resections in small

cohorts (179, 180). The ability to predict patients who will develop complications remains the holy grail in managing these complex patients. We suggest a novel score in chapter 5 to help predict patients at risk of major complications and anastomotic leak.

1.4.6.3. Medical treatment

Medical treatment for oesophageal cancer in the UK, except for neoadjuvant chemotherapy, has largely been used to treat patients unsuitable for potentially curative treatment. This is despite comparable results to surgery for some modalities.

Similar to many cancers some patient's cancers respond to treatment better than others. The ability to determine those that respond would be of value to guide more targeted and individualised therapy.

1.4.6.3.1. Primary radiotherapy

Radiotherapy was evaluated in patients who were not fit for surgery in an uncontrolled series of patients with SCC of the oesophagus and showed, at the time, similar survival at 5 years when compared to surgery (181). Since this time both the nature of oesophageal cancer, radiotherapy and surgical outcome has changed. No randomised clinical trials have been performed due to poor recruitment, despite attempts.

1.4.6.3.2. Neoadjuvant radiotherapy

The randomised control trials that have been performed looking at neoadjuvant radiotherapy compared to surgery alone have not demonstrated a survival advantage and have mainly been on patients with SCC (182).

1.4.6.3.3. Neoadjuvant chemotherapy

For fit patients presenting with operable oesophageal cancer the current standard of care in the UK is to advise neoadjuvant chemotherapy: 2 cycles of CF, followed by resection. This treatment pathway follows the optimum arm of the Medical Research Council OE02 study (94). The expected 2 year survival rate from this approach is 43% compared to 34% for surgery alone. A further predominately UK trial looked at perioperative chemotherapy and showed a significant improvement in survival compared to surgery alone (3).

This was in contrast to the findings of a North American study that concluded no survival benefit at two years when neoadjuvant chemotherapy was used compared to surgery alone (148).

1.4.6.3.4. Neoadjuvant chemoradiotherapy

Some trial's looking at neoadjuvant chemoradiotherapy have shown no statistically significant survival benefit when compared to surgery alone (149, 183). Despite this, neo-adjuvant chemoradiotherapy is employed in many countries based on the results of randomised clinical trials that estimate favoured survival data (184, 185). The trials

overall have showed improved pathological response and improved R0 rates with improvements in survival (186). Concern still arises from the potential for increased operative mortality following chemoradiotherapy, leading to the early termination of the FFCD 9901 trial.

A further trial is planned in the UK to see if radiotherapy should be added to neoadjuvant chemotherapy as the survival advantage over the current standard, neoadjuvant chemotherapy, is not yet evident for patients with AC of the oesophagus (187, 188).

1.4.6.3.5. Postoperative chemoradiotherapy

A USA intergroup trial showing improved survival with the addition of adjuvant chemoradiotherapy when compared to surgery alone. This approach has been adopted in the USA (189). The UK has largely failed to follow this approach claiming suboptimal surgery may have confounded the results with additional concerns over toxicity (82).

Postoperative chemoradiotherapy is frequently offered to patients with incomplete surgical resection margins in the UK. However this approach has not shown survival advantage and often patients are unable to tolerate further therapy (190, 191).

1.4.6.3.6. Primary chemoradiotherapy

The combination of radiotherapy and concurrent chemotherapy has lead to long term survival at 5 years of 26% an outcome comparable to surgery (192-194). The relative role of surgery versus a non-surgical combined modality approach remains undefined. The majority of trials confer advantage over other medical modalities but clinical trials of surgery versus chemoradiotherapy would be useful but have not been possible due to poor recruitment (195). The promise has been shown in unblinded studies of neoadjuvant chemoradiation with approximately 25% of those undergoing therapy achieving a complete response and improved survival compared with historical controls (196-198).

1.4.6.3.7. Chemoprevention

Extensive observational data show that non steroidal anti-inflammatory drugs (NSAIDs) are associated with a 50% or greater decrease in oesophageal cancer (45, 199) (200, 201). The exact mechanism of any chemopreventive effect is unclear and no randomized controlled trial has confirmed that this observed association is causative. However, given the poor prognosis associated with oesophageal cancer, the potential use of chemoprevention has been suggested in patients with BO. This is currently being assessed by the AspECT trial looking at the use of aspirin in BO patients despite previous studies showing no benefit (45, 46).

Initially after the introduction of PPIs, multiple studies showed that PPI therapy did not lead to reliable regression of Barrett's (202-204). Recently, longitudinal cohort studies

of patients with BO have suggested that the use of PPIs may be associated with a decreased risk of dysplasia in BO patients (205, 206).

In practice PPIs are used in these patients for both symptom control of GORD and the potential decreased risk of dysplasia despite the mechanism not being clear. The real long term effects of PPIs are not yet known but no negative effects have so far been identified.

1.4.7. Prognosis

At presentation more than 50% of patients have unresectable or metastatic disease. The stage at presentation for those that are resectable and the five year survival rate is summarised in table 1-3 for those with R0 resection.

Table 1-3: Stage of presentation and 5 year survival for patients presenting with resectable Oesophageal Cancer (124, 207, 208)

Stage	Presentation	5 year Survival
0	1-10%	>95%
I	13-20%	50-80%
IIa	14-27%	30-40%
IIb	7-16%	10-30%
III	40-54%	10-15%

Patients with metastatic disease (stage IV) who are treated with palliative chemotherapy have a median survival of less than a year (209).

1.5. The immune response

The immune response describes the defence of the body from pathogens by the complex interaction of various cells and molecules from the innate and adaptive arms of the immune system.

1.5.1. The innate immune system

Innate immunity targets harmful agents that have not been encountered before. This leads to a rapid, intense and generic response which is mediated by both cellular and humoral elements.

1.5.1.1. Cellular elements

1.5.1.1.1. Phagocytes

Macrophages and neutrophils are the main cells tasked with engulfing and digesting foreign material.

Phagocytosis leads to the release of cytokines and the acute inflammatory response which attracts immunological cells to the site. The phagocyte induces cell death using superoxide anion, nitric oxide, defensins, lysozyme, proteolytic enzymes (210) and presentation of pathogen derived antigens to T cells from the adaptive arm of the immune system.

1.5.1.1.2. Natural killer cells

Natural killer (NK) cells are lymphocytes which express cluster of differentiation (CD) 56 and/or CD16 but do not express CD3 and are crucial for innate immunity. NK cells cause cell death and release cytokines without specific sensitisation. They kill infected cells and cells that do not express major histocompatability complex class 1 molecules. To prevent normal tissue from initiating this process they are regulated through the expression of surface NK Receptors (NKRs) (211).

An activated NK cell kills by apoptosis through the initiation of the capsase cascade. This is partly mediated by the release of perforin which creates pores in the target cell's membrane so that pro-apoptotic factors like granzyme B can move into the cell (212). NK cells also produce tumouricidal cytokines and are either cytotoxic or cytokine producing depending on their expression of CD56 and CD16 (213, 214).

1.5.1.1.3. Gammadelta (γδ) T cells

γδ T cells are different from αβ T cells as they respond to antigen without a need for its presentation with MHC molecules. The T cell receptors (TCR), in this subclass, recognise a range of epitopes, reducing the need for clonal expansion when abnormal tissue is found.

These cells probably play a role in the removal of stressed or damaged cells. It has been noted that mice deficient in this cell type are predisposed to the development of tumours (210, 215).

1.5.1.2. Humoral elements

1.5.1.2.1. Cytokines, chemokines and acute phase proteins

Cytokines are low molecular weight proteins which are released by T helper (Th) cells, NK cells, mast cells, stromal cells and monocytes. There are several groups including interleukins, tumour necrosis factors (TNFs) and interferons (IFNs) which induce an immune response locally. Typical actions include the control of cellular differentiation, the upregulation of receptors, the initiation of an inflammatory response, anti-tumour and anti-viral activity, direct cytotoxicity and the priming of the adaptive immune response. Chemotactic cytokines attract cells, while interleukin (IL) 1 and IL-6 cause the release of acute phase proteins.

Acute phase proteins such as C-reactive protein (CRP) coat non-self material, opsonising them and fixing complement (210). Other acute phase proteins include coagulation proteins, complement proteins and albumin and are described as positive or negative depending on whether they increase or decrease inflammation.

1.5.1.2.2. Complement

Complement is an enzyme system of plasma proteins which mediate opsonisation, chemotaxis, leucocyte activation, lysis of foreign cells, antibody responses, immunological memory, the disposal of immune complexes and the clearance of apoptosed cells.

1.5.2. The adaptive system

The adaptive immune system deals with pathogens which evade innate responses and produces a more specific response to foreign body invasion. As with the innate immune system, both humoral and cell-mediated elements contribute.

1.5.2.1.1. Cellular elements

1.5.2.1.1.1. B-cells

B-cells develop in the bone marrow, during development they undergo rearrangement of their genes which code for the B-cell receptor (BCR). On encountering its corresponding antigen, BCR binding signals the B-cell to internalise the antigen, process it and express it bound to human leukocyte antigen (HLA) class II molecules. B-cells may develop further into plasma cells, which can clonally expand and produce identical antibodies in large quantities (210, 216) in response to antigens.

1.5.2.1.1.2. Human leukocyte antigen

HLA is the human form of major histocompatability complex (MHC) and its gene group on chromosome 7 encoding for three classes of molecules.

1.5.2.1.1.2.1. HLA class I region

HLA class I itself is a heavy chain 44KDa polypeptide molecule consisting of three globular helical domains (α1, α2, and α3) which are expressed on the cell surface and insert into the cytosol through the cell membrane. Its heavy chain is always associated with a β2microglobulin (β2m) chain and three different class I antigens (-A, -B and -C) are expressed, each of which displays marked allelic variation between individuals. HLA class I is expressed on nearly all cells and allows presentation of intracellular peptides associated with abnormal cellular conditions. These peptides are processed by the proteosome and are transported by transporters associated with antigen processing (TAP) 1 and 2 through the endoplasmic reticulum and Golgi apparatus to the cell surface. During this process it is bound to β2m and arrives at the cell surface as an HLA class I antigen. This peptide-HLA complex then becomes a target for cytotoxic T-cells (217).

1.5.2.1.1.2.2. HLA class II region

HLA class II molecules are made up of two helical domains (α1+α2) and two beta sheets (β1+β2). It is associated with B-cells, dendritic Cells (DCs), macrophages and thymic epithelium under normal circumstances with DP, DQ and DR subclasses. Its role

in antigen presenting cells (APCs) is to present processed foreign antigen to Th cells (217).

1.5.2.1.1.3. Dendritic cells

Cells which can present antigen in the context of HLA class II to Th cells are called professional APCs and include the macrophages, B-cells and DCs. DCs come from the bone marrow and are divided into two subsets: myeloid (mDC) and plasmacytoid (pDC). This is on the basis of their differential expression of surface markers like TLRs, CD8a, CD11b and CD11c. pDCs are found in blood and secondary lymphoid tissue and secrete large amounts of IFNγ in response to pathogens. mDCs are in the skin and gastrointestinal tract, areas where they are likely to come in contact with foreign antigen. Stimuli such as apoptosis, necrosis, inflammation and the presence of other immune system cells can activate mDCs, causing them to start maturation and migration to the lymph nodes. When mDCs arrive at lymph nodes, those presenting self antigens are deleted, suppressed or rendered anergic which allows the peripheral tolerance of self. However, when foreign antigen is being presented, the co-stimulatory molecules expressed on mature DCs cause naïve T cells to become activated, facilitating a primary immune response which causes the maturation of these T cells through the release of cytokines (218).

1.5.2.1.1.4. T cells

The two main types of T cell are the CD8+ (Tc, Cytotoxic T-Lymphocytes, CTLs) and the CD4+ (Th). Both come from bone marrow but undergo most of their development in the thymus where they differentiate from CD8+ CD4+, double positive cells, into CD8+ or CD4+ cells. This process is driven by an individual cell's strength of reaction when presented to HLA class I or class II molecules bound to self antigen in the thymus. Cells which do not react with HLA of either class are deleted, positive selection, and those which overreact to HLA are either deleted or become regulatory T cells (Tregs) in order to prevent autoimmunity, negative selection.

The surviving naïve CD8+ and CD4+ cells are the ones which recognise HLA class I or HLA class II respectively and do not express a TCR for self antigen (210). These lymphocytes are then ready to recognise non self antigens via their TCR, either presented on HLA class I or II molecules and so becoming activated and ultimately killing the cell. The TCR-antigen interaction triggers T cell division with some T cells becoming memory cells and possess the cell surface molecule CD45R0.

1.5.2.1.1.4.1. CD8+ cells

These recognise antigen presented in conjunction with HLA class I and so recognise and deal with the abnormal intracellular processes responsible for such peptide presentation within all cells. Its cytotoxic mechanism is the same as NK cells (217).

1.5.2.1.1.4.2. CD4+ cells

Naïve T cells are thought to require two signals for their activation with a lack of the second signal leading to a state of anergy. The first is normally TCR binding to an HLA-peptide complex on the surface of a DC in the periphery. For CD4+ cells, the second signal is the CD28 molecule binding to co-stimulatory molecules on the surface of APCs. Depending on the nature of the pathogen, the resulting clonal expansion of CD4+ cells produced from the naïve cell may produce a pool of either Th1 or Th2 cells. Th1 cells produce cytokines which help cellular immunity such as IFNɣ and IL-2 while the Th2 subset produces cytokines which aid humoral immunity. The resultant response to pathogen depends upon the balance of each pool as the cytokine responses antagonise each other (217).

1.5.2.1.1.4.3. Regulatory T cells

CD4+ CD8+ naïve T cell progenitors, not apoptosed or positively selected in the thymus, are thought to undergo differentiation into Tregs. These are mainly CD4+ cells and are characterised phenotypically by the expression of the transcription factor Forkhead box p3 (Foxp3). Their role involves the suppression of excessive or inappropriate immune responses (hypersensitivity/autoimmunity) and the dampening of the immune response once the original stimulus has been dealt with (217, 219).

1.5.2.2. Humoral elements

1.5.2.2.1. Antibodies

Antibodies or immunoglobulins bind the antigen of a particular foreign organism and initiate immune responses specifically directed against them. The antibody consists of two identical protein light chains and two identical protein heavy chains, represented as a “Y” shaped molecule, the stem of which is called the Fragment crystal line (Fc) unit and each arm of which is termed a Fragment antigen binding (Fab) unit. The Fc unit binds effector molecules while the Fab units have regions which are different between antibodies in order to bind specific antigens.

Antibodies come in 5 classes, IgG, IgM, IgE, IgA and IgD. Each Fc unit is different with each triggering different effector mechanisms. Each fixes complement when bound to antigen. Effector cells express Fc receptors for particular classes of antibody and antibodies trigger specific immune responses through both cell mediated and humoral routes. Each specific antibody originates from a single B-cell genetically predetermined to recognise a specific antigen (210).

Table 1-4: Types of immune cells, CD markers expressed and function

Immune cell	CD markers	Function
T lymphocytes	CD3	Cell mediated immunity.
T helper cells	CD4	Stimulate B-cell growth and differentiation. Activation of macrophages and CD8+ cells.
Cytotoxic T cells	CD8	Cell mediated killing of cells.
B cells	CD20	Production of antibodies, antigen presentation.
Memory T cells	CD45R0	Rapid expansion to produce effector cells.
Natural killer cells	CD56	Cytotoxic killing via apoptosis or cell lysis.
Macrophages	CD68	Phagocytosis and stimulation of lymphocytes.
T regulatory cells	FOXp3	Suppress activation of the immune system and maintain tolerance to self antigens.

1.5.3. The immune system and cancer

In Hanahan and Weinberg's the hallmarks of cancer the characteristics of cancer cells were put forward. They described that the development of cancer depended on six essential changes, or hallmarks, in cell physiology that together govern malignant growth. These were: self sufficiency in growth signals, resistance to inhibitory growth signals, evasion of apoptosis, limitless replicative potential, angiogenesis, and tissue invasion and metastasis (220). Each of these represents a breach of the patient's defence mechanism. Recently Hanahan and Weinberg have proposed four new hallmarks including inflammation and the evasion of the immune system (221).

1.5.3.1. Immunosurveillance

The concept of the immune system influencing the growth of cancer, the theory of immunosurveillance, was first put forward by Burnet and colleagues. They suggested that cancer cells were constantly being produced and being continually removed by the immune system at the subclinical level (222).

The theory was tested by comparing the frequency of spontaneous cancers in over 10,000 inbred mice presumed to be immunoincompetent compared to their wild type counterpart. No difference in frequency was found and the theory initially was rejected (223). However it seemed that the conclusions had been made using an imperfect model of immunocompromise. Later it was found that individuals lacking NKT, T and B-cells, perforin or IFNɣ were independently linked to increased tumour activity indicating important roles for them in a revised theory of immunosurveillance (224).

Evidence for the presence of immunosurveillance in humans has been gathered from the opportunistic observations of transplant patients, the comparison of clinical outcome with the presence of immune cells within the tumours and the study of tumour reactive antibodies in serum and their tumour associated antigens (TAAs) (224).

A review of patients receiving renal transplants showed a 3- to 4-fold increase in non-viral cancers at sites such as colon and lung and a two-fold increase in the risk of malignant melanoma (225, 226).

The type (CD4+, CD8+, CD45R0 and FOXP3+), location (tumour and stroma), functional orientation and density of immune cells in the tumour microenvironment and their association with clinical outcome are well documented in colorectal cancer (227-229). While the presence of CD8+ cells have been independently associated with improved prognosis in OAC (230). Similar findings have been shown for other cell types such as NK cells and the immune infiltrate was shown to vary in different tumour types (231).

Cells of the adaptive immune system specific for antigens found to be restricted to particular cancers have been discovered, showing the immunogenicity of tumour cells. The cancer testis antigen (CTA) New York oesophageal squamous cell carcinoma antigen (NY-ESO-1), which is restricted to a limited variety of normal tissues, has been found in cancers at sites where it would not normally be expressed and its upregulation as well as the production of specific CD4+ and CD8+ cells has been reported (232).

1.5.3.2. Immunoediting

The concept of immunosurveillance increasingly has been superseded by a broader model of “immunoediting”, a refinement of its predecessor aimed at explaining observations such as failure of immunosurveillance in clinically apparent tumours (224). Immunoediting states that the immune system interacts with the tumour mass in order to edit it rather than simply to destroy it, the immune system providing a Darwinian selection pressure favouring the propagation of cells with less immunogenic phenotypes. In this way tumours may be produced which ultimately grow successfully in spite of a functioning immune system. Evidence for this in murine models comes from the observation that tumours with a deficiency in perforin rapidly grow but remain immunogenic enough to be rejected when transferred to wild type mice. In contrast, when perforin functions normally the more immunogenic types are destroyed and these tumours are not rejected when transfered to mice with a competent immune system (233).

The theoretical model of immunoediting envisages three states in which tumours may reside namely: elimination, equilibrium and escape (234).

1.5.3.2.1. Elimination

The elimination state is the same as immunosurveillance as the result is the death of the tumour cell. The theoretical process begins with the development of a tumour cell which interacts abnormally with its environment due to its underlying hallmarks of cancer. This leads to the attention of the innate and adaptive immune system resulting in tumour cell death. NK cells and macrophages would then be synergistically activated leading to apoptosis and a slowdown in proliferation and angiogenesis.

Following tumour cell destruction by these cells, antigen would become available for presentation to the adaptive immune system via DCs which would migrate to the lymph nodes and stimulate the production of tumour specific CD4+ and CD8+ cells, leading to

tumour cell killing and the release of more anti-tumour cytokines. This process would occur every time a microscopic tumour arose and becomes less efficient with age or in immunosuppresed individuals (235).

1.5.3.2.2. Equilibrium

The equilibrium phase equates to a stage of tumour development where the tumour population has been incompletely eliminated. During this stage the inherent genetic instability of cancer would mean that certain cells within the population would be immunogenic and be removed, whereas other less immunogenic cells would be more successful and propagate.

It is thought that this phase would be the longest. A clinical example is highlighted in the long time between the development of genetically unstable BO and the development of invasive cancer. In addition if this undetectable process were actively under way in transplant donors, it might explain the relatively rapid development of non-viral cancers in the immunosuppressed recipients (234).

1.5.3.2.3. Escape

The final state of immunoediting would be an escape phase where the tumour mass would evolve to the point where it continues to grow despite the presence of a functional immune system.

A number of mechanisms known to occur in human cancers might underlie immunological escape such as loss of HLA class I expression, down regulation of tumour antigens, a lack of co-stimulation, the presence of immunosuppressive cytokines, apoptosis of T cells, the effects of Tregs, IFNɣ insensitivity (236) and MDSC function. It is likely that tumours develop strategies to escape both the innate and adaptive arms of the immune system.

1.6. Molecular Mechanisms in Pathogenesis

OAC is thought to develop through a series of somatic mutations or epigenetic changes allowing for several of the hallmarks of cancer to develop. This often leads to a survival benefit on the mutated cells and to a subsequent clonal expansion at the expense of the other cells. Chronic inflammation is thought to be the precipitating factor in the progression to OAC. In OAC, the inflammation is thought to be triggered by acid and bile reflux into the oesophagus as this is the strongest risk factor for the development of BO. The precise molecular mechanisms are not fully understood yet a variety of molecules have been proven to be involved (237).

Evidence from Next-Generation Sequencing studies confirms a causal link between GORD and OAC. Dulak and colleagues have performed exome and whole genome sequencing of 149 OAC tumor-normal pairs, finding a mutational spectrum unique to OAC (A>C transversions, predominantly in non-coding areas and less-expressed genes,

especially at AAG trinucleotides) suggesting that it is attributable to GERD and possibly as a result as oxidative damage (238). New insights, such as these, into the genetic landscape of OAC will make it possible to determine the precise carcinogens responsible for mutagenesis. For example, is gastric acid the driver of genome wide A>C transversions? Or, as has been suggested previously, are bile salts required for this effect (239), and is mutation in oesophageal epithelial cells a direct effect of the refluxate or mediated by the inflammatory response in the microenvironment? In vitro and in vivo experiments to answer these questions are now required and are important when one considers the relationship between the rise in PPI use and OAC.

1.7. Tumour associated antigens

The search continues for TAAs to direct the human immune system against cancer. Human TAAs can be classified, based on their expression pattern, into two major groups: unique antigens and shared antigens, with shared antigens further divided into tumour-specific antigens, differentiation antigens and over-expressed antigens.

Unique antigens result from point mutations in defined genes potentially implicated in tumour transformation. As these mutations are usually not shared by tumours from different patients, such tumour-specific antigens may only play an important role in the natural anti-tumour immune response of individual patients.

In contrast, shared antigens are present on some tumours from different patients and histological types. For example, CTAs are silent in healthy adult tissues, except testis and placenta, but active in a variety of cancers and therefore can be considered as tumour-specific. This specificity is useful for the purposes of cancer vaccine development (240). Differentiation antigens are also expressed in the normal cells of the same tissue from which a tumour develops. Antigens of this last group represent specific markers for a cell lineage (carcinoembryonic antigen, CEA), prostate specific antigen (PSA) and are not tumour-specific. Their use as targets for cancer immunotherapy may result in auto-immunity towards the corresponding normal tissue (e.g. vitiligo in the case of melanocytes). The third group of shared antigens, such as Human Epidermal growth factor Receptor 2 (Her-2), comprise antigens expressed in a wide variety of normal tissues and are overexpressed in tumours. The low level of expression in normal tissues should not cause autoimmune damage, although a threshold is frequently difficult to define.

1.7.1. Cancer testis antigens

1.7.1.1. Cancer testis antigen classification

About 100 CTAs grouped in over 40 families have been identified so far. The first identified CTA, the melanoma-associated antigen (MAGE) gene, MAGE-A1, was

characterized in 1991 through a newly developed methodology for identifying tumour antigens based on tumour-specific CTL recognition (241).

Further analysis of the MAGE-A family revealed twelve closely related genes clustered at Xp28 (242). Other clusters of MAGE genes were identified such as MAGE-B and MAGE-C as well as B antigen (BAGE) and G antigen (GAGE). Tumour-antigen genes structurally different from MAGE were also discovered.

A new methodology, based on the screening of cDNA expression libraries with antibodies from patient's serum (SEREX), led to the identification of several categories of CTA such as synovial sarcoma X chromosome (SSX), synaptonemal complex protein 1 (SCP1) (243) and the highly immunogenic cancer antigen, NY-ESO-1 (244, 245). 22 CTA families are encoded on the X chromosome (CT-X antigens).

Recently a new classification has been suggested due to the use of a variety of definitions in the literature. The key element of the classification of CTAs proposed by Hofmann *et al* based on their mRNA expression profile in testis and other tissues defines three groups: testis restricted; testis brain restricted and testis selective. The classification aims to provide improved understanding of their biological role (246). It serves to classify genes into perhaps more clinically useful subgroups. Both testis restricted and testis/brain restricted CTAs are potentially more useful as targets for immunotherapy due to their lack of expression in normal tissue, and expression limited to immunopriveleged sites. Testis selective CTAs, if used as targets, may induce autoimmunity, depending on their level of expression in normal tissue, but nevertheless may serve as useful biomarkers of disease progression.

1.7.1.2. Cancer testis Antigen expression

Proteins of the CTA families are expressed in a many malignant neoplasms. Bladder cancer, lung cancer, ovarian cancer, hepatocellular carcinoma, oesophageal SCC and melanoma frequently express CTAs. These antigens were rarely observed in renal cancer, colon cancer, gastric cancer and certain leukaemias. In normal tissues, specific CTA expression is restricted to testis germ cells (247) while within the placenta they are expressed in the trophoblasts (248, 249). As these cell types do not express HLA class I molecules, specific gene expression will not result in the presentation of antigenic peptides.

The expression of CTAs varies between tumour types. A summary of CTA expression in the oesophagus is shown in table 1-5. To date, analysis of CTA expression in oesophageal tumours has mostly focused on SCC (250). Different studies demonstrate that CTAs can be highly expressed within tumour tissues (251) but rarely homogeneously in all tumour cells (247), indicative of intaclonal variability in CTA expression in a given cancer.

The induction of CTA expression is most likely related to promoter demethylation (252, 253). All CTA genes examined reveal methylated CpG islands within gene

promoters in normal somatic tissues, resulting in genes being silenced (252). In contrast, in germ cells, CTA gene promoters are less CpG methylated (254). During tumour growth, global DNA demethylation and gene-specific demethylation is a known occurrence and is a plausible mechanism for inducing CTA expression in tumours.

1.7.1.3. Cancer testis antigen function

Knowledge regarding the functions of CTAs in germ and tumour cells is far from comprehensive. The majority of non-X CTAs display functional roles in spermatogenesis and fertilisation (255-257). The biological role of CTAs, positioned on the X chromosome (CTA-X), is less well understood, but have been examined in tumorigenesis, mainly by the use of yeast two-hybrid studies (258, 259). The data suggest that some MAGE family members may be involved in cell cycle control and apoptosis (260).

The profile of CTA expression in molecular studies points towards a clear involvement of CTAs in cancer development (261), with underlying mechanisms yet to be fully defined. CTA expression patterns however have also been exploited for a prognostic relevance of cancer patients, with variable scope (262-267).

1.7.1.4. Immunogenicity

Immunogenicity in cancer patients in part is evoked by short peptide CTA epitopes (268). They are presented by HLA class I molecules on the tumour cell surface for recognition by CTLs and by HLA class II molecules on the surface of APC in the case of Th cell mediated immune responses.

Identification of these epitopes recognised by the immune system is essential to facilitate development of tumour specific vaccines. The immunogenicity of CTAs is variable and not all have documented immune responses (269). NY-ESO-1 is a highly immunogenic CTA with spontaneous and coordinated humoral and cell-mediated immune responses in a high percentage of patients with NY-ESO-1 expressing tumours. Serum anti-NY-ESO-1 antibody has been detected in 13% of oesophageal SCC cancer patients (270). It has been further noted that more than 90% of patients with circulating anti-NY-ESO-1 antibodies also develop a specific CTL response that is absent in patients with undetectable anti-NY-ESO-1 antibodies (271).

CTAs due to their immunogenic capability and restricted expression are ideal targets for immunotherapy and biomarkers in cancer patients and this is explored further in chapter 9.

Table 1-5: Cancer testis antigen frequency (%) of expression in oesophageal specimens

CT gene family/identifier	Normal	BO	OAC	Gastric AC	Oesophageal Cancer Unknown	Ref
MAGEA/CT1	-	-	15-57	-	-	(272)
MAGEA1/CT1.1	0	0	18-20	15-33	-	(273-277)
MAGEA2/CT1.2	0	0	20-33	33	-	(273, 274)
MAGEA3/CT1.3	0	n/a	38	42	-	(273, 275)
MAGEA4/CT1.4	-	-	25	25	-	(244,246)
MAGEA6/CT1.6	-	-	17	-	-	(273)
BAGE1/CT2.1	-	-	0	-	-	(273)
MAGEB1/CT3.1	-	-	-	0	-	(278)
MAGEB2/CT3.2	-	-	-	0	-	(250, 278)
GAGE/CT4.1	-	-	13	-	22	(272, 273)
SSX1/CT5.1	0	-	-	0-23	-	(243, 250)
SSX2/CT5.2	0	-	-	0-3	-	(231,238)
SSX3/CT5.3	-	-	-	0	-	(243)
SSX4/CT5.4	0	-	-	27-33	-	(231,238)
SSX5/CT5.5	-	-	-	0	-	(243)
NY-ESO-1/CT6.1	0	-	-	0-12	57	(272)
LAGE1/CT6.2b	0	-	-	17	-	(275)
MAGEC1/CT7.1	-	-	-	14	-	(275)
MAGEC2/CT7.2	-	-	-	6	-	(275)
SYCP1/CT8	0	-	-	6-7	-	(250, 275)
SPANXC/CT11.3	0	-	-	0	-	(279)
OYTES1/CT23	-	-	-	0	-	(256)
MMA1a/CT25.1a	0	-	-	-	-	(280)
CAGE/CT26	-	-	-	89	-	(281)
D40/CT29	-	-	-	0	-	(282)
HCA661/CT30	-	-	-	0	-	(275, 283)
NYSAR35/CT37	-	-	-	-	8	(255)
FTHL17/CT38	-	-	-	-	0	(284)
NXF2/CT39	-	-	-	-	13	(284)
TAF2Q/CT40	-	-	-	-	0	(284)
TDRD1/CT41.1	-	-	-	-	10	(284)
TEX15/CT42	-	-	-	-	20	(284)
FATE/CT43	-	-	-	7	-	(285)
TPTE/CT44	-	-	-	0	-	(285)
OIP5/CT86	-	-	-	59	-	(286)
SCRN1	-	-	-	71	-	(287)

1.8. Biomarkers

A clinical biomarker can be defined as a characteristic that can be objectively measured or evaluated as an indicator of normal biological process, pathological processes or a response to a therapeutic intervention (288). The aim is to improve and possibly change clinical management.

In the case of BO and OAC patient's biomarkers could predict risk by population screening, predict risk of progression to dysplasia or OAC in BO patients, predict response to neoadjuvant chemotherapy or preventive treatments or predict prognosis and disease recurrence.

Despite large numbers of reports on tumour biomarkers in general, the number of clinically useful markers is small (289) and currently the only biomarkers in use for BO and OAC are dysplasia and the standard pathological parameters such as the TNM classification. There are currently no predictors of response to neoadjuvant therapy. Despite frequent discovery of potential biomarkers, the lack of translation has led to the development of strategies for their detection, reporting and validation.

1.8.1. Strategies for detection of biomarkers

A phased approach has been adopted for the development of therapeutic drugs (290) and for prevention trials (291). More recently a phased approach has been suggested for the detection of biomarkers.

This approach, suggested by the Early Detection Research Network, describes five phases from preclinical exploratory, clinical assay and validation, retrospective and prospective phases before the biomarkers impact on clinical practice is quantified (292). Within each phase the recommendations aim to improve study design. In addition they aim to enable the formulation of criteria so biomarkers can be assessed to see if they should progress through the phases of development.

1.8.2. Reporting of biomarkers

Similar to the CONSORT (293) guidelines for randomised control trials and the STARD(294) statement for studies of diagnostic accuracy the REMARK guidelines have been developed to suggest information regarding biomarkers.

The REMARK guidelines suggest information that should be included regarding study design, preplanned hypotheses, patient and specimen characteristics, assay methods and statistical analysis as well as the presentation of results and discussion (289).

1.9. Immunotherapy and oesophageal cancer

The aim of immunotherapy is to enable the patient's immune system to achieve tumour cell killing by two broad strategies. Active immunotherapy is aimed at inducing host immune responses to eradicate cancer. This is achieved by generating long-lived immunity targeted to TAAs through vaccination and by non-specific immunomodulation, such as by using bacterial products or targeting negative regulatory receptors or molecules that prevent the development of the tumour immune response. Passive immunotherapy is defined as administration of an immunologically active agent to a patient, such as monoclonal antibodies of which Rituximab in lymphoma is a notable example (295).

Currently there are no immunotherapies used in the routine treatment of oesophageal cancer. There are however a number of UK trials assessing the utility of

monoclonal antibodies or small molecules with or without chemotherapy in patients unsuitable for radical treatment.

1.9.1. Vaccination

The principle behind active vaccination strategies is to challenge the immune system with TAA for processing by APCs. TAA-derived epitopes are then presented via HLA class I/II to recuit and activate CD8+/CD4+ T-cells to mediate tumour. The advantages of such a strategy as compared to traditional drug-based treatments include potentially reduced toxicity, obviating drug resistance, and generating immunological memory (296). The potential risk of autoimmunity can be overcome by the careful selection of the appropriate tumour target antigen.

Currently one phase 1 trial looking at vaccination against CTA derived peptides in oesophageal SCC patients has shown promising disease control rates with no serious side effects in a small series (297). It must also be recognised that vaccine strategies face several hurdles: (A) they must overcome an immunosuppressive tumour environment with HLA class I loss or infiltration of the tumour by Tregs; (B) the patient's immune system recognise the cancer as self rather than foreign, leading to tolerance issues.

1.9.2. Cytokines

IFN-α and IL-2 have been the principle cytokines used as immunotherapies against cancers predominately in melanoma and renal cell carcinoma patients. The central role of IL-2 is in coordinating the inflammatory response and its association with regression in human cancers (298). The use of cytokines as drugs in oesophageal cancer has been limited to studies in cell lines. The use of IFN-ɣ has been shown in oesophageal SCC cell lines to promote apoptosis (299).

1.9.3. Adoptive T cell transfer

A passive method of initiating a cell mediated response involves the transfer of modified T cells. This has not been extensively explored in oesophageal cancer patients and the potential has only been addressed in small numbers in mainly SCC patients. This study evaluated intra/peri-tumoural infiltration of autologous tumour-cells stimulated by cytotoxic T lymphocytes and showed increased infiltration with T effector cells and increased anti-tumour response (300).

1.9.4. Monoclonal antibodies and small molecules

Passively immunotherapy may be achieved through the use of monoclonal antibodies (mAbs) that specifically target antigens associated with cancer or its progression. They can act either by recruiting effector immune cells (principally NK cells) for antibody-dependent cytoxicity or through blocking pathways that are integral to the hallmarks of cancer such as growth regulation, angiogenesis, inflammation, cell cycle

control, apoptosis and metastatic potential (220). The use of mAbs in haematological malignancies (notably Rituximab) is routine but their efficacy in oesophageal cancers is currently under study in clinical trials in the UK.

1.9.4.1. Epidermal growth factor receptor inhibitors

EGFR is a member of the ErbB receptor tyrosine kinase family that includes ErBb2 (HER-2), ErBb3 and ErbB4 (301). Over expression of EGFR, as wild type or with mutations, occurs in many human tumours, including OAC (92%) (302). Expression of EGFR is associated with poor prognosis and advanced disease. Currently two methods for targeting EGFR are under investigation: mABs and tyrosine kinase Inhibitors (TKIs). The results show a potential increase in efficacy of chemotherapy in SCC oesophageal patients (303) and modest response rates in OAC patients (304).

There are currently two UK trials, SCOPE-1 and REAL-3, looking at the use of monoclonal antibodies, Panitumumab and Cetuximab, with or without chemotherapy in patients unsuitable for radical treatment. Gefitinib, a TKI, is being assessed in recurrent oesophageal cancer within the UK as part of the COG trial.

1.9.4.2. Human epidermal growth factor receptor 2

HER-2 is a proto-oncogene localised on chromosome 17q and encodes a transmembrane tyrosine kinase growth factor like EGFR. Amplification of HER-2 antigen has been shown to occur in approximately 30% of breast cancer patients and correlates with agressiveness of the tumour (305). It is now common place for a mAB, trastuzamab, to be included in therapy for HER-2 positive tumours in combination with chemotherapy. This has led to improved overall survival in these patients (306). Its appears to act by downregulating of HER-2 expression, initiating cell mediated cytotoxicity and promoting apoptosis (307).

It has been shown that HER-2 overexpression in oesophageal SCC and AC occur at similar frequencies to those seen in breast cancer patients (308). In SCC of the oesophagus, overexpression was associated with extramural invasion and poor response to neoadjuvant chemotherapy, while in patients with OAC an association was found with invasion, metastasis and overall survival (309). Trastuzmab, a monoclonal antibody that targets HER-2, has been assessed in a small number of patients with locally advanced tumours that overexpress HER2, showing no additional toxicity but little survival benefit (308, 310).

1.9.4.3. Vascular endothelial growth factor

Angiogenesis regulates tumour growth and metastasis. Vascular endothelial growth factor (VEGF) is a strong and specific angiogenic factor regulating both normal and pathologic angiogenesis (311). Its expression in oesophageal cancer in approximately 30-60% of cases correlates with advanced stage and poor OS (312). In

Barrett's dysplasia the glandular epithelium secretion of VEGF-A may prove to be a potential marker or target to stop progression (313, 314). Bevacizumab a recombinant humanised mAb inhibits VEGF directly; it's use in combination with chemotherapy in 47 oesophageal and gastric adenocarcinoma patients reported improvements in time to disease progression but the study was limited by its comparison with historical controls. The incidence of complications was high in this series with 6 of 25 patients experiencing thrombo-embolic events, two perforations and one near perforation (315). Bevacizumab is currently being assessed in the UK as part of the STO-3 study, with and without chemotherapy in GOJ and stomach cancer patients.

1.10. Immune markers in oesophageal cancer

Virchow first described the association of lymphocyte infiltration with solid tumours and it is becoming increasingly recognised that inflammation plays a major role in tumourgenesis (316). In both SCC and AC of the oesophagus, chronic inflammation is thought to be the precipitating factor (19). Immune responses, whether systemic or within the tumour microenvironment, are increasingly implicated as a determining factor in tumour progression. The most comprehensive studies have been in colorectal cancer correlating infiltration of the tumour with tumour infiltrating lymphocytes (TILs) with improved survival (227).

Despite the evidence of tumour-host immunological interaction, our understanding of this process is incomplete. It has been suggested that inappropriate host inflammatory response may result in cancer growth, invasion and metastasis via a plethora of mechanisms including subversion of host immune responses, upregulation of cytokines and inflammatory mediators, inhibition of apoptosis, promotion of angiogenesis and damage of DNA (317). The presence of immune cells in the tumour microenvironment highlights the role of the immune response in the tumour niche, the challenge being to identify the precise nature of the anti-tumour response. The nature of this response however may reveal systemic and local immunological biomarkers that could be evaluated together for making decisions regarding patients' prognosis and response to treatment (318, 319).

We examine the current status of the immunological response in oesophageal cancer and BO before exploring potential biomarkers in chapter 7 and 8.

1.10.1. Innate immune system

1.10.1.1. Tumour-associated macrophages

Tumour associated macrophages (TAMs) exhibit both pro- and antitumour effects, depending on specific tumour states (320). TAMs comprise 2 distinct types based on their function: M1 are proinflammatory with antitumour activity and M2 are immunosuppressive, angiogenic and protumour (321-324).

Macrophages as CD68+ cells have been assessed in heterogenous groups of patients with gastric, GOJ, OAC and oesophageal SCC with conflicting association found with CSS (325). Other groups have studied the TAM infiltrate in oesophageal SCC patients and have shown independent association of high macrophage index and OS. These findings are not universal, with other groups finding no association with TAM infiltration and survival (326-330).

1.10.1.2. Natural killer cells

NK cells have been shown to be linked with improved prognosis when found in greater numbers infiltrating tumours of the gastrointestinal tract (331-333). In one study of patients with SCC of the oesophagus limited numbers of NK cells showed no correlation with prognosis but other reports have shown a positive association (334, 335). As yet no studies have reported on NK infiltration in OAC.

1.10.1.3. Cytokines

The levels of proinflammatory cytokines (IL-1B and IL-8) and Nuclear Factor Kappa-B (NFk-B) respectively have been shown to be increased along the presumed progression of oesophagitis through Barrett's to OAC. There was also an association between these two parameters in OAC tissue (336, 337). NFk-B has numerous roles in cell regulation so the association may not be directly related.

IL-12 and IL-18 expression levels have been assessed by two groups who have both shown that their levels correspond to increasing stage of disease. However, it was not associated to OS in patients with SCC (338, 339).

IL-6 has been shown to inhibit apoptosis and may therefore be involved in the transformation and progression to cancer (340). IL-6 is increased in BO compared to normal tissue from the same patients. It was concluded that this may lead to apoptosis resistance and so lead to a higher chance of transformation (341).

1.10.2. Adaptive immune system

1.10.2.1. Tumour infiltrating lymphocytes

The type (CD4+, CD8+, CD45R0 and FOXP3+), location (tumour and stroma), functional orientation and density of immune cells in the tumour microenvironment and their association with clinical outcome are well documented in colorectal cancer (227-229).

In SCC of the oesophagus, TILs, CD4+ and CD8+, have been associated with improved survival (326, 342, 343). Intratumoural CD8+ T cell infiltration has been identified to offer favourable prognosis in a study looking at both SCC and OAC histological subtypes, including 37 patients with AC of the oesophagus (230). In contrast a study looking at 106 patients who underwent surgery only for AC of the oesophagus found association with TILs and survival on univariate analysis only (344). Further

confirmatory studies have shown significant associations with reduced local non-specific inflammatory response and worse survival in both OAC and oesophageal SCC (325, 326, 345-347). In addition the assessment of memory T cells, CD45RO+, in early stage OAC patients revealed independent associations with survival (347). The differences in findings may well be due to different methodologies employed in assessing TILs as well as the differences in the study populations. Quanifying TILs and immunophenotyping when not standardised is a major hurdle in comparing between studies. This emphasises the need for uniformity in reporting if the relevance of TILs is to be translated into the clinical practice in OAC.

Examining the functional significance of CD8+ cells, Schumacher *et al* analysed a mixed population of oesophageal tumours for the occurrence of IFN-Ɣ secreting CD8+ cells and found some but not all T-cells to be activated (230). The study highlights that CD8+ cells are able to infiltrate the tumour but not all are able to mount an antitumour response, and heterogeneity of response, where single T-cell families can vary in functional capacity. In terms of active immunotherapy the presence of active intratumoural CD8+ cells is encouraging and it will be informative to know whether these CTL are antigen specific.

Immunosuppression can impact on T-cell populations, and has been observed in Barrett's patients when compared to normal and reflux oesophagitis patients indicating reduced T and B cell function as well as IL-2 production (348). The exact means of T cell suppression in tumours as a whole is not fully understood. Evidence in oesophageal cancer has included direct immunosuppressive factors from the tumour and with suppressive rather than cytotoxic T cells (349, 350).

1.10.2.2. Regulatory T cells

Tregs suppress the host immune response and may associate with tumour growth. Few studies have examined FoxP3+ Tregs in oesophageal cancer. Two studies have found no association with the infiltration of FoxP3+ Tregs with survival in oesophageal SCC (351, 352). One study found no overall association of FoxP3+ Tregs with survival in OAC. They found association with reduced intratumoural FoxP3+ Treg counts with increasing age and stage of disease and with survival on univariate analysis but not on multivariate analysis (344).

1.10.2.3. Dendritic cells

Dendritic cells have been shown to be present in BO with increasing numbers in oesophageal AC (353, 354). The study did not look at the prognostic significance or function of these cells yet their increasing number indicates their potential involvement in the pathogenesis. This is inferred by the known central ability of dendritic cells to function as professional antigen cells in normal immune responses in cancer immunity (316, 355).

1.10.2.4. HLA class I

Down regulation of MHC class I expression has been shown to correspond to a poorer prognosis in oesophageal SCC and to be down regulated in OAC and BO (356-358). The molecular mechanisms underlying abnormal HLA class I phenotypes in oesophageal cancer has not been explored in date.

1.10.2.5. Humoral response

Moons et al suggest that the humoral response is the more important factor in the progression from Barrett's to oesophageal AC, based on their finding of increased frequencies of Th2 effector cells with an increase in Ig E expressing plasma cells (359). This apparent association is not confined to oesophageal cancer but is evident in other cancers (360).

1.10.3. Peripheral blood inflammatory markers

A number of studies have assessed the association of different markers of systemic inflammation in patients with solid tumours with survival mainly focusing on patients with inoperable or metastatic disease (361-374) (Table 1-6). Preoperative neutrophil count (363), lymphocyte count (363), platelet count (364, 365), albumin (366), CRP (362, 367, 368), neutrophil/lymphocyte ratio (NLR) (362, 369, 370), platelet/lymphocyte ratio (PLR) (362, 371), prognostic nutritional index (PNI) (362, 372) and Glasgow prognostic score (GPS) (373, 374) and modified Glasgow prognostic score (mGPS) (362) have been associated with changes in survival in oesophagogastric cancer patients. A common feature of all these studies was the lack of uniformity in the survival outcome chosen as well as inconsistency in the cut-off value for the proposed marker. Taking NLR as an example different studies found cut-off values ranging from 2 (369) to 5 (370) to be associated with survival. There are also conflicting reports on the association of NLR and survival in radically treated oesophageal cancer patients (370, 375). Rashid *et al* found no association between preoperative NLR and survival (375) whereas Sharaiha *et al* found preoperative NLR to be an independent predictor of both DFS and OS after oesophagectomy (370).

1.10.4. Summary

There is increasing evidence of oesophageal cancer being immunogenic, eliciting both humoral and cell-mediated reactions of both the innate and adaptive arms of the immune system. So far the primary cancer studied has been SCC rather than OAC. But evidence of immunoediting has been shown in other gastrointestinal tumours including AC. A better understanding of the mechanisms by which OAC tumours avoid immunosurveillance will lead to potential ways to optimise immunotherapy strategies in AC the more prevalent tumour now in the UK.

Table 1-6: Studies investigating the impact of peripheral blood values and scores on prognosis of patients with oesophageal and gastric cancer

Year	No.	Cohort	Values studied	Findings
1982 (363)	204	G (stage IV)	Neutrophil count Lymphocyte count	<6.0 improved OS (p<0.001) >1.5 improved OS (p<0.001)
1998 (369)	55	G (stage I)	NLR	<2 improved OS (p=0.0022)
2001 (367)	262	O (curative)	CRP	<5 improved OS (p<0.0001)
2002 (365)	369	G (stage I)	Hb Platelet count	NS for OS <400 improved OS (p<0.0001)
2003 (368)	150	O (curative)	CRP	<1 improved OS (p=0.049)
2004 (364)	374	O (curative)	Platelet count	<293 improved OS (p=0.009)
2003 (366)	356	O (curative)	CRP Lymphocyte % Albumin	<5 improved OS (p=0.0285) ≥25 improved OS univariate (p=0.0486) ≥38 improved OS univariate (p=0.0486)
2005 (376)	67	O (stage I-IV)	CRP	>6mg/l improved DFS (p=0.016)
2006 (377)	291	O (curative)	CRP	<5 improved OS (p=0.063)
2006 (378)	120	O, G (stage I-III)	Hb, WCC, Lymphocyte % Platelet count, Albumin CRP	NS NS NS <10 improved CSS (p<0.001)
2006 (374)	258	O, G (inoperable)	GPS	Improved CSS for active treatment (p<0.001) NS for CSS for supportive treatment
2008 (373)	65	O, G (inoperable)	WCC Lymphocyte % GPS	NS for CSS Univariate only for CSS (p=0.030) Improved CSS (p<0.05)
2008 (379)	1220	G (stage IV)	Neutrophil count Lymphocyte count NLR	NS for OS NS for OS <2.5 improved OS (p=0.019)
2009 (380)	220	O, G (stage I-IV)	CRP	<5 improved OS (p=0.031)
2010 (372)	248	G (curative)	PNI	≥46 improved OS (p=0.009)
2010 (381)	1027	G (Curative	NLR	<4 improved OS (p<0.001)
2010 (371)	168	G (local)	Lymphocyte count Platelet count NLR PLR	>1.5 improved OS (p=0.03) NS for OS <2.56 improved OS (p=0.001) <160 improved OS (p=0.006)
2010 (382)	357	G (stage I-IV)	NLR CRP Albumin	<2.2 improved OS (p<0.001) NS NS
2010 (375)	294	O (stage I-IV)	NLR	NS with OS
2010 (383)	70	O	CRP WCC	< improved OS (p=0.027) NS
2010 (384)	217	O (stage I-IV)	mGPS ECRS CRP	Improved CSS (p<0.001) NS (univariate p<0.001) NS (<5 univariate p<0.001)
2010 (385)	217	G (stage I-IV)	CRP Albumin	<10 improved CSS (p<0.001) ≥35 ns
2011 (386)	112	O, OGJ (curative)	WCC, NLR, PLR Neutrophil count Lymphocyte count Platelet count mGPS	NS for CSS NS for CSS NS for CSS NS for CSS mGPS 0 improved CSS (p<0.001)
2011 (370)	295	O, OGJ (curative)	Neutrophil count Lymphocyte count NLR	Univariate OS (p=0.001) and DFS (p=0.04) Univariate OS (p=0.005) <5 improved OS and DFS (p<0.0001)
2011 (387)	495	O (curative)	mGPS	0 improved DFS and OS (p<0.001)
2011 (362)	874	O, G (stage I-IV)	mGPS NLR PLR PI PNI	mGPS 0 improved CSS (p<0.001) <5 improved CSS (p<0.001) <150 improved CSS (p<0.001) PI 0 improved CSS (p<0.001) ≥45 improved CSS (p<0.001)
2011 (388)	293	G (stage III-IV)	NLR	<2 OS (p=0.002); <3 DFS (p<0.001) improved
2011 (389)	204	G (stage I-III)	CRP	<5 improved OS (p<0.0001)

2. Chapter 2: Materials and methods

This chapter describes the process for gaining ethics, general techniques and specific experimental and analytical methodologies used to achieve the aims of this research.

2.1. Research governance

In order to perform these studies, the principles laid out in 2001 and revised in 2005 of the department of health research governance framework for health and social (RGF) care needed to be met as well as the local research and development (R&D) general policy.

2.1.1. Ethics

This sets out key principles to ensure integrity and quality, informed consent of participants, confidentiality, voluntary participation, avoidance of harm of participants and to avoid conflicts of interest in the research. In essence, it outlines the legal and moral responsibility of a researcher so that patients enrolled in a study are protected by the principles of good clinical practice (GCP).

2.1.1.1. Review bodies

The submission of proposals for review was recently streamlined. This meant the proposal was entered into the Integrated Research Application System (IRAS). This enables a single form to be sent on to all the relevant review bodies. Documents include protocol and study plan (Appendix 1), proof of funding, sponsorship, peer review, patient information sheets, consent forms, researcher curriculum vitae, site specific form and application form. For this project relevant review bodies included the National Health Service (NHS) research and ethics committee (REC), R&D and the National institute for health and research (NIHR).

2.1.1.2. Ethical committee approval

The application for ethical approval for the study was submitted via IRAS to the Southampton and South West Hampshire REC (B) and was approved on the 21st May 2009 (REC 09/H0504/66). Mr Tim Underwood and Dr Andrew Bateman attended.

2.1.1.3. Ethical considerations

The main ethical considerations for this project involved the storage of patient tissue and confidentiality of patient information.

2.1.1.3.1. Consent

To ensure informed consent was obtained prior to using a patient's data and tissue, sufficient information regarding the study was provided in a patient information sheet (Appendix 2). Patients were also provided with time to assess the implications for

themselves and others and the chance to ask questions before consenting to taking part in the study (Appendix 3).

With regard to the use of historical tissue of historical patients, all patients had consented to the use of their tissue in research as part of the consent process for their treatment. It was felt to be inappropriate to contact patients or bereaved relatives as this may upset the family or patient. Approval was therefore sought on the basis of their previous consent for their tissue to be used in research.

2.1.1.4. Human tissue act

The human tissue act 2004 replaced the human tissue act 1961, the anatomy act 1984 and the human organ transplants act 1989 and its implementation is overseen by the human tissue authority (HTA). The reason for its introduction were that it had become commonplace for human tissue to be used without consent (390).

To enable the research to proceed meant using the local Experimental Cancer Medicine Centre (ECMC) run tissue bank approved by the HTA.

2.1.1.5. Pseudoanonymisation

To ensure confidentiality to the patients whilst not losing the benefits that come from a prospectively maintained database, the LREC required for the database to be pseudoanonymised and maintained on an encrypted server.

2.1.2. Research and development

The R&D departments were set up in individual NHS organisations to ensure implementation of the RGF for each study being carried out in their organisation. Final approval to commence the research was given on the 18th August 2009 (RHM CAN0649). The R&D department ensures ethical approval for the study is in place. The study has suitable sponsorship and for this study the sponsor is University Hospital Southampton Foundation Trust (UHSFT). The sponsor is the individual or organisation responsible for ensuring that researchers have the capabilities and resources in place as well as providing insurance for the study. A requirement of the sponsor is for research studies to undergo peer review which was performed by Professor JN Primrose.

2.2. Materials

2.2.1. General materials

Table 2-1: Reagents, media and solutions

Reagent	Cat no:	Supplier
Agarose	1356-500	Fischer scientific
1 Kb Plus DNA ladder	10787-026	Invitrogen
RNase free water	129112	Qiagen
Blue loading buffer	10816-015	Invitrogen
Ethidium bromide	15585-011	Invitrogen
Bovine serum albumin (BSA)	15561-020	Invitrogen
Phosphate buffer saline	10010-031	Invitrogen
FACS Buffer (PBS, 0.5% w/v BSA, 0.1% w/v NaN_3)	n/a	n/a
RPMI 1640	32404-014	Invitrogen
Testis human RNA	636533-Lot7100353	Clontech
TRI reagent®	T9424	Sigma Aldrich
BEGM	CC-3170	Lonza
DMEM	A14430-01	Invitrogen
Fetal calf serum	10082-147	Invitrogen
Tris-acetate-EDTA	n/a	n/a
Isopropanol	401570	Invitrogen
HotStarTaq DNA polymerase	203203	Qiagen
Sodium pyruvate	11360-070	Invitrogen
Penicilin and streptomycin	15070063	Invitrogen
L-Glutamine	21051-040	Invitrogen
RNeasy mini kit	74104	Qiagen
SuperScript™ II Reverse transcriptase	18064-022	Invitrogen
QIAquick gel extraction kit	28704	Qiagen
BigDye Terminator v3.1	4337455	Applied biosystems
anti-human CD326 (EpCAM) PE	12-9326-41	eBioscience
GoScript reverse transcription system	A5000	Promega
*p*GEM-T vector system I	A3600	Promega
QIAprep Spin miniprep kit	28794	Qiagen
Taqman Universal Mastermix II no UNG	4440040	Applied biosystems
Customised TaqMan Array 64 format	4346800	Applied biosystems
Xgal	B4252	Sigma
IPTG	I5502	Sigma
SP6 (TATTTAGGTGACACTATAG)	Q5011	Promega
T7 (TAATACGACTCACTATAGGG)	Q5021	Promega
EpCAM-PE	347198	Biolegend
HLA-A*02-FITC	343304	BD bioscience
IgG_1-FITC isotype control	556028	BD bioscience
CD3-APC.Cy7	300318	Biolegend
CD4-FITC	120-000-798	Miltenyi
CD8-PERCPCy5.5	341050	BD bioscience
Calcein-Pacific blue	34958	Invitrogen
PASD1 tetramer-PE	n/a	In house
MAGEA3 tetramer-PE	n/a	In house
CTAGE1 tetramer-PE	n/a	In house
EBV tetramer-PE	n/a	In house
HIV tetramer-PE	n/a	In house
CTAGE1 peptide (FLWRSFRSV)	n/a	Peptide protein research
PASD1 peptide (QLLDGFMITL)	n/a	Peptide protein research
MAGEA3 peptide (KVAELVHFL)	n/a	Peptide protein research

Table 2-2: Plastic ware and other materials

Plastic ware	Cat no:	Supplier
Pestle	7339-901	Argos technologies
Filter and non-filter Pipette tips	Various	STARLAB
Cryos	122 2XX	Greiner bio-one
Universals	Various	STARLAB
Bijoux	Various	STARLAB
Pastelletes	Various	STARLAB
Parafilm	I3080-5075	STARLAB
PCR tubes 0.2ml	I1402-8108	STARLAB
PCR tubes 0.5 ml	I405-8108	STARLAB
Falcon tubes various	Various	SARSTEDT
Cell culture dishes	430167	Corning
Microtubes	Various	Sarstedt
Multi screen filter plates (ELISPOT)	MAIPS45190	Milipore
Cell culture flasks and wells	Various	Corning

Table 2-3: Experimental apparatus

Apparatus	Location	Supplier
HERA Cell	Tenovus	Heraeus
Nanodrop spectrometer	CSD	Thermo fischer scientific
GS1 thermal cycler	Tenovus	G-Storm
Ultraviolet light box	Tenovus	Amplirad
OMNI PCR workstation	Tenovus	Astec
FACS Aria flow cytometer	IIR	BD biosciences
FACS CantoII flow cytometer	CSD	BD biosciences
ABI3130xl DNA sequencer	Tenovus	Applied biosystems
Techne Dri-Block DB-2A	Tenovus	Techne
MS2 Minishaker	Tenovus	IKA
Gel electrophoresis tank	Tenovus	Hybaid
Gene power supply GPS 200/400	Tenovus	Pharmacia
Multifuge 3SR+	Tenovus	Thermo scientific
Freezer -80ºC	Tenovus	Labcold
accuSpin Micro R	Tenovus	Fischer scientific
Elispot plate reader	Tenovus	Pet
JB aqua 12 plus water baths	CSD	Grant
Microscope	CSD	Leica
Culture hoods Mars safety class 2	CSD	Labogene
GelDoc-it imaging system	CSD	UVP
Incubator Glaxay 170 S	CSD	New Brunswick
7500 thermal cycler	CSD	Applied biosystems
7900 thermal cycler	IIR	Applied biosystems

Table 2-4: Software used for analysis

Software	Version	Supplier
SPSS	19.0	IBM
CIA	2.2.0	UoS
Excel	12.0	Microsoft
OlyVIA	2.4	Olympus
FACSDiva	6.0	BD biosciences
MacVector	7.2.3	MacVector
UV vision works ls	6.3.3	UV vision works

2.2.2. Cell lines

OE19, OE21, OE33, HET1A and Flo-1 cell lines were a gift from Mr Tim Underwood. This panel of gastro-oesophageal cell lines were selected as they have been recently been shown to be representative of gastro-oesophageal cancer unlike many OAC cell lines (391).

Table 2-5: Human gastro-oesophageal cell lines used in thesis

Cell line	Source	Notes
OE19	Mr TJ Underwood	72yr old male with AC of gastric cardia/GOJ. UICC Stage III. Grade II differentiation.
OE21	Mr TJ Underwood	74yr old male with SCC of oesophagus. UICC stage IIA. Grade II differentiation.
OE33	Mr TJ Underwood	73yr old female with AC of lower oesophagus with BO. UICC stage IIA. Grade III differentiation.
HET1A	Mr TJ Underwood	SV-40 immortalised human oesophageal cell line.
FLO-1	Mr TJ Underwood	68yr old male with AC of lower oesophagus.

2.2.3. Patient material

Patient material was collected for the study (REC 09/H0504/66) at either a weekly endoscopy list at UHSFT or at the time of surgery. Suitable patients were highlighted at the weekly MDT meeting and given appropriate patient information sheets in clinic by Donna Sharland, cancer nurse specialist (Section 12.2: Appendix 2). All patients attending for the weekly endoscopy list were sent patient information sheets prior to their attendance. Patients were then recruited and consented to take part in the study (Section 12.3: Appendix 3). Tissue was stored with the help of the ECMC tumour bank staff following the study protocol (Section 12.1: Appendix 1). This ensured that all material was isolated and prepared for storage at -80ºC as quickly as possible. Biopsy and tumour samples were prepared by using a sterile scalpel to ensure they were of appropriate size, ≤ 30 mg. The samples were then snap frozen in liquid nitrogen in the endoscopy unit or theatre. Ethylenediaminetetraacetic acid (EDTA) and clotted blood samples required preparation in the laboratory to enable isolation of peripheral blood mononuclear cells (PBMCs) and serum before they could be stored. PBMC and serum isolation was performed by the ECMC team using Ficoll-paque plus and centrifugation. The Ficoll-sodium metrizoate solution leads to differential migration of the components of the blood during centrifugation.

Clinical management of patients included discussion at a specialist multidisciplinary team meeting (MDT). Standard staging investigations included endoscopic ultrasonography, high-resolution computed tomography, integrated fluorodeoxyglucose positron emission tomography/computed tomography (PET-CT) and staging laparoscopy where indicated. Patients considered suitable for potential surgical resection with tumours staged as T2 N0 M0 or above were considered for neoadjuvant chemotherapy. Neoadjuvant chemotherapy consisted of three 21-day cycles of ECF (Epirubicin 50mg/m^2, Cisplatin 60mg/m^2, both intravenously on day 1 and protracted venous infusion 5-FU 200mg/m^2 per day) or ECX (Epirubicin 50mg/m^2, Cisplatin 60mg/m^2, both intravenously on day 1 and Capecitabine 625mg/m^2 orally twice daily for 21 days) or EOX (Epirubicin 50 mg/m^2 i.v. bolus and Oxaliplatin 130 mg/m^2 i.v. infusion over 2 hours on day 1, Capecitabine 625 mg/m^2 orally twice daily for 21 days).

Surgery was performed at UHSFT after initial staging or 4-6 weeks following neoadjuvant chemotherapy. A repeat CT scan was performed for those that received

chemotherapy to assess their response to chemotherapy and disease operability. Types of oesophagogastrectomies included Ivor Lewis, left thoracoabdominal with or without cervical anastomosis and transhiatal oesophagogastrectomy or minimally invasive oesophagogastrectomy (MIO) either 2 stage (MIO-2) or 3 stage (MIO-3) in accordance with recommendations arising from the consensus statement from the Association of Upper Gastrointestinal Surgeons and the Association of Laparoscopic Surgeons for introduction of MIO (392).

Data recorded included demographics, tumour characteristics, resection type and histopathological analysis of the surgical specimen. TNM-7 was used to report tumour stage after analysis of pathology reports(393). Pathological tumour clearance ("R"-status) was determined according the Royal College of Pathologists system. Pathological response to chemotherapy was assessed using the tumour regression grade (TRG) system developed by Mandard *et al* (83, 394).

Patients were routinely followed-up for 5 years post surgery according to the following protocol: 2-4 weeks post-discharge, 3 monthly for 1 year, 6 monthly for 2 years and yearly thereafter. Patients were also seen on an as required basis if symptomatic. Recurrence of disease during follow-up was defined as the first site or sites of recurrence with radiological or pathological confirmation. For assessment of disease free survival (DFS), recurrence was defined as time from operation to development of local, nodal (regional) and distant metastasis (whichever occurred first). Overall survival (OS) was defined as time from operation to date of death.

2.3. Methods

2.3.1. Cell culture

Cell culture is a technique to enable the in vitro proliferation of animal or human cells in a nutrient medium for extended periods of time. It provides a ready supply of DNA, RNA and proteins and enables the cells to be manipulated if required. All cell lines were maintained in logarithmic growth in a humidified cell incubator (HERA Cell, Heraeus) at 37ºC and 5% CO_2 (10% CO_2 for FLO-1 cells). Complete growth medium of Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% w/v heat inactivated fetal calf serum (FCS), 2.0 mM L-glutamine, 50 I.U./ml of penicillin (100 U/ml) and 50 µg/ml of streptomycin and 5 mls of 1.0 mM sodium pyruvate was used for the culture of OE19, OE21 and OE33 cells. Bronchial epithelial cell growth medium (BEGM) was used for the culture of HET1A cells. Dulbecco's modified eagle medium (DMEM) supplemented with 2 mM glutamine, 10% w/v heat inactivated fetal calf serum (FCS) and 50 I.U./ml of penicillin and 50 µg/ml streptomycin was used for the culture of FLO-1 cells.

Cells were fed by changing media every 3-4 days until confluent. Cells were passaged when confluent using trypsin. Old medium was aspirated from the flask. Cells were washed with warm PBS and then warm trypsin was added and swirled around to

coat all cells. The flask was then placed in the incubator for 10 mins. Cells were displaced from the flask surface and media was added to inhibit the enzyme reaction before the cells were damaged. The cells in solution were then added to a 15 ml falcon and centrifuged at 1,500 rpm for 5 mins. The supernatant was removed and discarded. The remaining cell pellet was then resuspended in media and the cells counted. Either the cells were placed in new culture flasks with appropriate volume of media or upto 1 × 10^7 cells in solution were centrifuged ready for extraction of RNA.

2.3.2. Cell count

Viability of cell lines and PBMCs was assessed using trypan blue exclusion prior to their usage. A coverslip was placed on the middle of the hemocytometer. 10 µl of cells and 10 µl of trypan blue solution were placed in a well of a 96 well plate. The solution was mixed by pipetting and tapping the plate to mix. 20 µl was placed on the hemocytometer by allowing a drop held at the end of the tip to be taken under the coverslip by capillary action. The hemocytometer was then placed on the stage of the microscope, and the grids were located. A 1 mm^2 area was then counted to give between 30 and 300 cells/mm, if there were too many cells the sample was diluted. Viable cells were counted in 1 mm^2. Dead cells stained entirely blue whereas viable cells did not stain at all or only had a blue ring around their edge. Cells were counted in a total of three different 1mm^2 squares and then averaged. The number of cells per ml equalled the average number of cells per 1 mm^2 x 10,000 x dilution of sample (i.e. 2). Viability above 80% was deemed satisfactory.

2.3.3. CTA gene transcript expression in human cell lines and primary tissue

2.3.3.1. Tissue disruption and homogenisation

Oesophageal tissue was removed from -80 ºC storage and a disposable pestle used to grind the tissue while still frozen under liquid nitrogen. For cell pellets the lysis buffer was added immediately. 350 µl of lysis buffer, RLT with 10 µl/ml of β-Mercaptoethanol (β-ME), was added to the eppendorf containing the tissue or cell pellet. The solution was then homogenized using a syringe and needle. The lysate was passed through a 20 gauge needle attached to a sterile plastic syringe at least 10 times so as to produce a homogeneous lysate.

2.3.3.2. RNA isolation and purification

To enable gene transcription of CTA mRNA in cell lines and primary patient material, total RNA was isolated using a Qiagen RNeasy kit. The RNeasy procedure combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to

provide appropriate binding conditions and the sample is then applied to an RNeasy Mini spin column where the total RNA binds to the RNeasy silica membrane and contaminants are washed away.

The steps begin by adding 350 µl of 70% ethanol to the homogenised lysate and mixing well by pipetting. 700 µl of the lysate was then added to an RNeasy mini column and placed in a 2 ml collecting tube. The tube was closed and centrifuged for 15 s at 8000 × g (10,000 rpm). The flow-through was discarded and the collection tube reused. 700 µl of buffer RWI was loaded onto the RNeasy column and the tube closed before being centrifuged for 15 s at 8000 × g (10,000 rpm). The flow-through was discarded. 500 µl of buffer RPE (220 ml of 100 % ethanol was added to buffer RPE before use) was added to the RNeasy column and the tube closed before being centrifuged for 15 s at 8000 × g (10,000 rpm). The flow-through was discarded. 500 µl of buffer RPE was added again to the RNeasy column and the tube closed before being centrifuged for 2 mins at 8000 × g (10,000 rpm) to ensure the RNeasey silica-gel membrane is dry from ethanol. The RNeasy mini column was removed from the collection tube and placed in a new 1.5 ml elution tube and 30-50 µl of RNase free water was pipette onto the RNeasy silica gel membrane. The tube was closed and centrifuged for 1 min at 8000 × g (10,000 rpm). RNA was then quantified and stored at -80ºC in aliquots.

2.3.3.3. Reverse transcription for cDNA synthesis

Reverse transcription (RT) was performed on 10 µl (1ng-5µg) of total RNA using SuperScript™ II RT performed in the hood with incubations performed in a thermocycler. SuperScript™ II RT is a point mutant of moloney murine leukemia virus RT engineered to be RNase H negative. The lack of RNase H activity eliminates the competition with 5'-3' DNA polymerse activity resulting in higher yields of cDNA (395). A short sequence of deoxy-thymine nucleotides (Olgo (dt)) is tagged as a complementary primer to the poly-A tail and provides a free 3'-OH end that can be extended by reverse transcription.

1 µl of Oligo $(dT)_{12\text{-}18}$ (500 µg/ml), 10 µl of total RNA and 1 µl of dNTP mix (10 mM mix of dGTP, dTTP, dCTP, dATP) were added to a 0.2 ml nuclease free microcentrifuge tube. The mixture was heated to 65ºC for 5 mins and quick chilled on ice. This reaction denatures and incubates the RNA with the Oligo (dT) primer and as the mixtures is chilled, the primer anneals to the RNA. The contents of the tube were collected by brief centrifugation.

Following centrifugation a master mix of 4 µl of 5 × first-strand buffer, 2 µl of 0.1 M dithiothreitol (DTT) and 1 µl of RNase free water were added. The contents were mixed gently and incubated at 42ºC for 2 mins. The DTT is used to inhibit RNase activity by reducing disulfide bonds required by RNases for their stability (396).

The last step added 1 µl (200 U) of SuperScript™ II RT by gently pipetting to ensure the solution was well mixed before incubation at 42ºC for 50 mins to enable the

reverse transcription reaction. The reaction was then inactivated by heating to 70ºC for 15 mins. The first strand cDNA template was stored at -20ºC.

2.3.3.4. Nucleic acid quantification

mRNA and cDNA quantification was performed on a Nanodrop 1000 absorbance spectrophotometer.

1 µl of deionised water was loaded onto the pedestal and used as a reference to initialise the spectrophotometer and was then cleaned by wiping the bottom and top sample pedestal with a clean soft tissue. The machine was calibrated before the 1st and after the 10th nucleic acid sample using 1 µl of water to check the baseline and sample carry-over. 1 µl of DNA or RNA was used for each sample reading and between samples the pedestal was cleaned. DNA and RNA samples were quantified in ng/µl and the 260/230 and 260/280 ratios recorded for determining DNA and RNA quality. RNA samples were considered to be satisfactory when the 260/230 (absorbance of nucleic acid/absorbance of salts, solvents and protein) and 260/280 ratios (absorbance of nucleic acid/absorbance of protein) were approximately 1.8 - 2.2 and approximately 2.0 respectively. For cDNA samples the ranges were between 1.8 - 2.2 and approximately 1.8 (397). RNA samples were then frozen at -80°C and cDNA stored at -20°C, ready for use as RT-PCR templates.

2.3.3.5. Primer design

Primer design is important for successful PCR amplification. Primers are short single stranded DNA molecules. The following considerations were taken when designing the pairs of primers. The length of the primer should be between 18-30 base pairs (bp) to ensure that the primer is long enough for adequate specificity but short enough so that they can bind to the template at the annealing temperature. The primer melting temperature, Tm, is the temperature required to separate the double stranded DNA where the primer anneals to the DNA and should be between 55-72ºC. Primers with a high Tm can give rise to secondary annealing and too low a temperature will result in a low yield of PCR product. The Tm of each of the primers designed for each CTA should be within 5 ºC of each other. Primers should never have a base repeated four or more times in a row. Primers should have a G/C concentration of between 40-60% and have G or C bases within the last 5 bases of their 3' end. This helps to promote specific binding at the 3' end due to the stronger bonding of G and C bases.

The design of primers was carried out by first finding the protein coding CTA gene transcript using Ensembl (www.ensembl.org). The primers were designed to span introns and were complementary to the target DNA sequence so as the PCR product could be distinguished from genomic DNA (Table 2-6) and two sets of primers were required to perform nested PCR. For CTAGE1, an intronless gene, a no reverse transcriptase (RT)

control was run for positive samples to ensure the positivity was not down to genomic DNA. This afforded the least amount of template to be wasted.

Primers were also designed for certain CTAs to further delineate between splice variants of the gene and so primers were designed to cover areas of difference between the variants. The primers are listed in tables 2-7 and 2-8 and were designed inconjunction with Gavin Babbage.

Table 2-6: Cancer testis antigen polymerase chain reaction product sizes

CTA	Internal product size (bp)		Classification	Notes
	exons only	with introns		
CSAG2	258	416	Testis restricted	
MAGEA2	525	1424	Testis restricted	
MAGEC1	322	809	Testis restricted	
NY-ESO-1	601	1511	Testis restricted	
SAGE1	276	1026	Testis restricted	
SSX2	549	7090	Testis restricted	
CTAGE1	652	n/a	Testis/Brain restricted	No introns
GAGE1	244	3532	Testis/Brain restricted	
HORMAD1	379	7879	Testis/Brain restricted	
MAGEC2	464	1421	Testis/Brain restricted	
PAGE3	241	3292	Testis/Brain restricted	
PASD1	426	36895	Testis/Brain restricted	
BAGE	274	40552	Testis selective	semi nested ext-ext int-ext
FATE1	579	6721	Testis selective	
HAGE1/DDX43	432	2332	Testis selective	
LAGE/CTAG2	400	1244	Testis selective	
MAGEA3	904	2769	Testis selective	
SPAG9	606	33173	Testis selective	
β-actin	399	495	Control	

Table 2-7: External primers for reverse transcriptase polymerase chain reaction

Gene	External forward primer sequence (5'-3')	External reverse primer sequence (5'-3')	Supplier
MAGEC2	GGAGGCGCGAATCAAGTTAG	CTCACTGAATGAGCTCCATG	Sigma
MAGEA3	GAGATTCTCGCCCTGAGCA	GAATTCATAACATGCAGGATCAC	Sigma
MAGEA2	CTCAGGAGTTGATGACCTTG	GCAATTTCTGAGGACACTCTCC	Sigma
LAGE	GAGGCCCTGGCATTCCTGAT	CTCCTCCAGCGACAAACAATC	Sigma
HORMAD1	GAAGAGGCTTCTAGCAGTTTCAG	CATTCGTTCTCTCTCAGTGGTC	Sigma
HAGE	GATGACTTGAAGGATGGGGAG	CATTTCTCTTCCTCGGTGGTTAC	Sigma
GAGE	GTGAAGAACGCCAFFFAGCT	GTTTCCGTGGGGAAAGATTTAAG	Sigma
FATE	CTTACAAGAGAACAGCTGGTTGTG	CAAAGGGTGGCATCTCAGC	Sigma
MAGEC1	GGCATTTTGTGACGAGGATC	CTCTGGAGAGGATCCGAGGAG	Sigma
SPAG9	CAAGACAACTTGAGCTGAAAGC	CAGGTCAGTTCATCCACTTTTG	Sigma
SAGE1	CCAATGCATTGGATTCTTTC	CTCCTTGGTCTTTGCACTATCAC	Sigma
PASD1	CGAGAACTCCAGGTCCACTG	GATAGTTCTGCTTACGAAAACGTG	Sigma
SSX2	TTCAAGGCCACCCTCCCACC	ACATCTGGGGAGAGAGGAGGG	Sigma
PASD1-V_{1-2}	CCAATCTTCGGAGGCAGTG	CTCCAGGGTGCTTATGGTTG	Sigma
PAGE3	GAGGAAGGTGGACCGTAGAG	CTGCATGTATGGTTTCAGCTTG	Sigma
NY-ESO-1	CCCTGACCTTCTCTCTGAGAG	CTACAGAAACAAACATGTAAGCCG	Sigma
CTAGE1	CTTTGAAGAAACTGATTCATGCTG	GAAACATCCTATAGTCCTGTTC	Sigma
BAGE	GCTTACAGGACCAGGAGAAGG	GATAGTGGCTCCAAAGTGCTTAC	Sigma
MAGEA4	GCAGACAGGCCAACCGGAG	GTCGCCCTCCATTGCAATTG	Sigma
MAGEA8	GAAGCGGGCTCAGGGTCTG	GGCAGGTGACAAGGATGTAGG	Sigma
MAGEA9	GTTCTCGGGACAGGCTAACCAG	CTCAGCCACCTTCAATTTCAGTG	Sigma
B-actin	GGCATCGTGATGGACTCCGGTG	CTGCTGGAAGGTGGACAGCGA	Sigma

Table 2-8: Internal primers for reverse transcriptase polymerase chain reaction

Gene	Internal forward primer sequence (5'-3')	Internal reverse primer sequence (5'-3')	Supplier
MAGEC2	GAGGGACCTCCCACCATAGAG	GCTCTCGGTAAGATTTGGTATC	Sigma
MAGEA3	CTGACGTCGGCGGAGGGAAG	GGGATCCCCCAAGATACTGT	Sigma
MAGEA2	AGGTCAAGACAGGGGCCCCC	AACTCGGACTCCAGGTCGGG	Sigma
LAGE	GCAGGATGGAAGGTGCCC	CTGGCCACTGTGCTGGGA	Sigma
HORMAD1	GCCCAGGATCTACACAGTTAGTG	CAATCACCATCCTTAAAACCG	Sigma
HAGE	GCCTTTCAATGTTATCCTGAG	GGTTATATTCTTCAGATTGACGAAG	Sigma
GAGE	GACCAAGGCGCTATGTACAC	CCATCAGGACCATCTTCACAC	Sigma
FATE	GAAAACCAAGAGCACCTGGTG	GGCTGCTGGGAGATGAAAG	Sigma
MAGEC1	CAGAAAGCAGGAGTTGAAGACC	CAGGAGAACTCTGGGGAATC	Sigma
SPAG9	GTGATCAACTAGAATCCACAGCTC	CTCCTTCATCCACATCTCCTATTAG	Sigma
SAGE1	GTGACCCACCAGTTACAGTAATG	CTTAAGCCTTCTCGAGTTG	Sigma
SSX2	GTGTAATAAACGGGCCGAAG	CCACGTTCTGCTTCTCATCA	Sigma
PASD1	CCTGTGGTCTTTAGTGGCTTG	GGATAAGGCTGGAGATTCAGG	Sigma
PASD-1-V_1	CTCTCCCACATCCCAAGGAC	GAGGTATCTATCAATGGGCAGC	Sigma
PASD1-V_2	CTCTCCCACATCCCAAGGAC	GTCTGTGATCTCAGGCATGG	Sigma
PAGE3	CTCTAATCATCCAGTAGGGGCTG	CTCCAGATTTGGCAGAAATTCTC	Sigma
NY-ESO-1	GATGCTGATGGCCCAGGAGG	CGACAAACAAGCAGGCCCCC	Sigma
CTAGE1	CTTCAGACTGAACAAGCATCTTTG	CTCTTTCTTTGGTAATCTGATGGTC	Sigma
BAGE	TGGCTCGTCGTCTCACTCTGG	CCTCCTATTGCTCCTGTTG	Sigma
MAGA4	GAGGAGCACCAAGGAGAAGAT	CAGGCAGGTGACAAGGGTGTAG	Sigma
MAGEA8	GAGGTCAGGAGGCCCCAGAG	CCTGCATGCACTCAGAGGC	Sigma
MAGEA9	GAGCAGCACTGACGAAGACC	CTGAGCTGGGTCGACCGAG	Sigma
B-actin	CTGACTACCTCATGAAGATCC	CAGTGATCTCCTTCTGCATCC	Sigma

2.3.3.6. Polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) is a technique widely used for the amplification of DNA sequences from a template at low concentration (398). A DNA polymerase is used to amplify target DNA by in vitro enzymatic replication and the product is formed in an exponential fashion. The heat-stable polymerase Taq, originally isolated from the bacterium Thermus aquaticus, uses single stranded DNA as a template and DNA oligonucleotides as primers to initiate DNA synthesis. A positive control, human testis cDNA, was used due the known expression of CTAs in human testis. A negative control reaction was included with all reactions lacking only cDNA template.

2.3.3.6.1. Nested reverse transcription polymerase chain reaction

Nested RT-PCR involves two sets of primers being used in two successive runs of polymerase chain reaction, the second set amplifying a secondary target within the first run product so as to reduce unexpected products sometimes seen with conventional RT-PCR (399).

All RT-PCRs were undertaken with HotStarTaq DNA polymerase and predominately used the G-Storm GS1 thermal cycler with the setup performed in a PCR hood pre-illuminated with ultraviolet (UV) light. HotStarTaq DNA polymerase is inactive at ambient temperatures so prevents extension of nonspecifically annealed primers and primer-dimers formed at low temperatures during PCR setup and initial PCR cycle. The plastic ware and water used was Rnase and DNA free and also UV illuminated before use. UV illumination causes crosslinking of any contaminating DNA which ensures the DNA cannot be amplified.

Table 2-9: Mastermix for reverse transcriptase polymerase chain reactions

Component	Volume
10× PCR buffer (200mM Tris-HCl (pH8.4), 500mM KCl)	5 µl
50 mM $MgCl_2$	1.5 µl
10 mM dNTP Mix (dGTP, dTTP, dCTP, dATP)	1 µl
Forward primer (20 mM)	1 µl
Reverse primer (20 mM)	1 µl
HotStarTaq DNA polymerase	0.5 µl
DNA	1 µl (external primer reaction)
	5 µl (internal primer reaction)
PCR grade H_2O	to make upto 50 µl

In the first step cDNA is denatured by heating the RT-PCR reaction. This allows the polymerase and primers access to the exposed nucleotides. The reaction is then cooled to 58ºC which allows the primers to anneal to the correct sequence of DNA ready for extension. The reaction is then rapidly ramped to allow extension where the polymerase adds dNTPs along the region of the gene being amplified.

Table 2-10: Cycling conditions for reverse transcription polymerase chain reaction

Step	Temp (ºC)	Duration (mins)
1	94	15
2	94	1
3	58	1
4	72	1
5	29 times to step 2	
6	72	3
7	4	To hold

The polymerase will discontinue adding dNTPs when the reverse primer stop codon is reached. The newly amplified DNA goes back into the cycle which is repeated in this case 29 times. This results in an exponential increase in DNA copy number.

The RT-PCR reactions were performed using a nested approach with 1 µl of cDNA being used for the first RT-PCR reaction using the external set of primers. This was followed by 5 µl of the first RT-PCR reaction being used for a second RT-PCR reaction using the internal set of primers before being run out on agarose gel.

2.3.3.7. Agarose gel electrophoresis of polymerase chain reaction products

RT-PCR products were electrophoresed on 2% w/v agarose gel. 3 grams of agarose was weighed and dissolved in 150 mls of R0 water by heating in the microwave. The agarose solution was allowed to cool. 7 µl of ethidium bromide (EtBr) was added to the agarose solution before pouring into the gel former and left to set.

50 µl of the RT-PCR product was mixed with 4 µl of blue loading buffer and loaded on the gel. The loading buffer helps to monitor the approximate location of the RT-PCR product and loading of the wells. 3 µl of 1 kb plus ladder was loaded to the first lane of the gel to size the products and one lane left blank to ensure no contaminants in the agarose gel and ethidium bromide solution.

The RT-PCR products were electrophoresed for 30-60 mins at 80-100 volts dependant on the size of the RT-PCR products submerged in 1 × tris-acetate-EDTA (TAE) buffer. The gel was then visualised on a UV illuminator.

2.3.3.8. Quantitative reverse transcription polymerase chain reaction

Quantitative real time polymerase chain reaction (qRT-PCR) is used to amplify and simultaneously quantify a specific sequence in a cDNA sample so as to quantify gene expression. qRT-PCR is similar to RT-PCR except that the progress of the reaction is monitored by a camera in real time.

The taqman system used utilises a primer and a probe complementary to the gene of interest. The oligonucleotide probe is labelled with a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye. During polymerisation the probe anneals downstream from one of the primer sites and as this is

extended by Taq DNA polymerase the reporter dye is cleaved. The cleavage of the reporter dye separates the reporter dye from the quencher dye and increases the reporter dye signal. The cleavage also removes the probe from the target strand allowing primer extension to continue. Each PCR cycle allows primer annealing and primer extension with cleavage of further reporter dyes.

Reactions are quantified by the point in time during cycling when the fluorescence exceeds a given threshold. The more template of interest present, the quicker this happens. A Ct value is generated and refers to the number of cycles needed to generate a defined amount of fluorescence during the linear phase of PCR. Thus a high Ct value indicates less expression of the gene of interest.

The exponential nature of PCR means that small differences in starting template between samples may skew exponentially to give errors in interpreting absolute Ct values. Therefore a comparative method is often used to quantify gene expression relative to an endogenous control gene. Typical endogenous control genes, housekeeping genes, include glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or beta actin (B-actin) or 18S rRNA. However to make the comparative Ct method valid it is important that the efficiency of the endogenous control and target gene amplification must be approximately equal with the endogenous control being equal between the samples of interest. This requires a validation experiment. It is also suggested that more than one housekeeping gene is included to improve the reliability.

qRT-PCR was performed with the AB7900 fast qRT-PCR machine. Commercially available probes were chosen to provide a wide screen of CTAs from all three classes (Table 2-11). A Taqman low density array format was used using micro fluidic technology to enable 64 targets to be assessed in triplicate.

1000ng of cDNA was suspended and made up to 50 µl with nuclease free water and 50 µl of Taqman universal PCR master mix II. The tubes were vortexed to ensure the solutions were mixed and then centrifuged to eliminate bubbles. Each reservoir of the microfluidic card was then loaded with 100 µl of template-master mix solution as per the manufactures instructions. The cards were then loaded into buckets and centrifuged for 331 × g (1,200 rpm) for 1 minute. The cards were checked to ensure filling was complete and, if not, a further minute of centrifuging was required. The card was then sealed and the fill reservoirs trimmed. The card was then ready to load onto the PCR machine.

Table 2-: Cycling conditions for real-time polymerase chain reaction (7500 real time system)

Step	Temp (ºC)	Duration (mins)
1	50	2
2	95	10
3	95	0.25
4	60 (40 Cycles)	1

Table 2-11: Taqman probe assays for quantitative polymerase chain reaction

Gene	Assay Identification	Classification
GAPDH	Hs02758991_g1	Control
HMBS	Hs00609297_m1	Control
TBP	Hs00427620_m1	Control
CSAG2-3	Hs01680099_gH	Testis Restricted
CTAG1B	Hs00265824_m1	Testis Restricted
CXorf48	Hs00250428_m1	Testis Restricted
CXorf61	Hs02386421_g1	Testis Restricted
DKKL1	Hs00819324_mH	Testis Restricted
IGF2BP3	Hs00559907_g1	Testis Restricted
LY6K	Hs03988347_m1	Testis Restricted
MAGEA1	Hs00607097_m1	Testis Restricted
MAGEA2	Hs00606323_m1	Testis Restricted
MAGEB1	Hs00159005_m1	Testis Restricted
MAGEB2	Hs00427156_m1	Testis Restricted
MAGEC1	Hs00193821_m1	Testis Restricted
SAGE1	Hs00218707_m1	Testis Restricted
SSX2-2B	Hs00817683_m1	Testis Restricted
SSX3	Hs00602463_mH	Testis Restricted
TSPY1-4	Hs00413986_m1	Testis Restricted
TTK	Hs00177412_m1	Testis Restricted
XAGE5	Hs00793258_g1	Testis Restricted
CTAGE1	Hs00535737_s1	Testis/Brain restricted
CTNNA2	Hs01093122_m1	Testis/Brain restricted
GAGE1	Hs00275619_m1	Testis/Brain restricted
GAGE1,2,4,5,7,8,6,12,13	Hs00275620_m1	Testis/Brain Restricted
HORMAD1	Hs00611993_m1	Testis/Brain restricted
MAGEA9-9B	Hs00893224_m1	Testis/Brain restricted
MAGEC2	Hs00212255_m1	Testis/Brain restricted
MAGEC3	Hs00602633_g1	Testis/Brain restricted
PAGE3	Hs00418462_m1	Testis/Brain restricted
PASD1	Hs00542865_m1	Testis/Brain restricted
CT47	Hs00414930_m1	Testis selective
DDX43	Hs00218682_m1	Testis selective
FATE1	Hs00263795_m1	Testis selective
MAGEA10	Hs00377891_m1	Testis selective
MAGEA3	Hs00366532_m1	Testis selective
MAGEA4	Hs00365979_m1	Testis selective
MAGEA6	Hs00602508_m1	Testis selective
MAGEA8	Hs00377810_m1	Testis selective
NXF	Hs00276482_m1	Testis selective
PAGE4	Hs00199655_m1	Testis selective
PAGE5	Hs00373098_m1	Testis selective
PLAC1	Hs00222307_m1	Testis selective
SPAG9	Hs00187715_m1	Testis selective
SSX4	Hs00846047_mH	Testis selective
TAF7L	Hs00227589_m1	Testis selective
XAGE3	Hs00431071_m1	Testis selective

2.3.3.8.1. Relative expression analysis used for qRT-PCR

The relative level of gene expression of CTAs was compared between different oesophageal tissue relative to normal oesophagus normalising to endogenous controls using the delta delta Ct method (Figure 2-1).

$$2^{\Delta\Delta CT} = 2^{(\text{Ct of gene of interest} - \text{Ct of reference gene})_{\text{control sample}} - (\text{Ct of gene of interest} - \text{Ct of reference gene})_{\text{test sample}}}$$

Figure 2-1: $2^{\Delta\Delta CT}$ method used for the relative quantification of gene expression

2.3.4. Intraclonal cancer testis antigen expression

To examine intraclonal CTA expression, single cells were examined in HET1A, OE21 and OE33 cell lines. Single cells were obtained by flow cytometry deposition and analysed using nested RT-PCR and confirmed by DNA sequencing.

2.3.4.1. Single cell flow activated cell sorting

Single cells were sorted using flow activated cell sorting (FACS) by staining with anti-human CD326 (EpCAM) PE, a pan-epithelial marker, and using singlet gating. Cells were sorted into microcentrifuge tubes containing 10 µl of 1 × reaction buffer using the FACSaria and stored ready for mRNA extraction.

1×10^6 cells were centrifuged at 1,500 rpm for 5 mins and resuspended in FACS buffer. 100 µl was added to one FACS tube for unstained control with 900 µl stained with 50 µl of EpCAM-PE and placed in the dark for 30 mins. The cells were then centrifuged at 1,500 rpm for 5 mins and washed with 1 ml of FACS buffer and centrifuged for a further 5 mins at 1,500 rpm. The cells were then resuspended in 500 µl of FACS buffer ready for sorting.

2.3.4.2. mRNA extraction and cDNA synthesis reaction

The method utilises similar methodology to the, previously described, RNA extraction and cDNA synthesis. 1 × reaction buffer containing sorted cell was mixed with 2 µl Oligo dT primer, 9 µl of nuclease free H_2O and 6 µl of nonidet P-40 (NP-40). The NP-40 serves to lyse the cell. The mix is then heated to 70ºC for 5 mins to denature the template and chilled to 4ºC for 5 mins and then the template-primer mix is ready for reverse transcription.

For the reverse transcription reaction 0.5 µl of nuclease free H_2O, 6µl of 5 × reaction buffer, 4 µl of $MgCl_2$, 2 µl of dNTP and finally 0.5 µl of GoScript reverse transcriptase is added to the template-primer mix. The reverse transcriptase is added just prior to use as it is immediately active.

The reverse transcriptase is run at 25ºC for 5 mins to anneal the primer followed by 60 mins at 42ºC for the extension step. The reverse transcriptase is inactivated at 72ºC for 15 mins before being chilled to 5ºC to provide 40 µl of cDNA template for PCR.

2.3.4.3. Nested reverse transcriptase polymerase chain reaction

The same protocol was followed as for expanding all cells (Section 2.3.3.6.1). 4 µl of cDNA was used in the initial amplification with 6 µl of the first amplification being used in the second round with the internal primers.

2.3.5. Confirmation of PCR products by DNA sequencing

Although the size of the PCR products may confirm appropriate sized DNA products have been amplified, DNA sequencing is required to confirm the PCR product is actually the desired product. PCR products were sequenced using dideoxynucleotide (ddNTP) chain termination (400).

2.3.5.1. Gel extraction of polymerase chain reaction products

For fluorescent based sequencing of PCR products, removal of unused primers, nucleotides, enzymes, agarose, ethidium bromide and impurities from the DNA sample was performed using a QIAquick gel extraction kit.

The DNA band was excised using a clean scalpel. 300 µl of QG buffer was added to the DNA band in an eppendorf and incubated at 50ºC on a heat block for 10-15 mins until the gel slice had fully melted. The colour of the mixture is normally yellow indicating a pH ≤ 7.5, so no sodium acetate was added. A pH ≤ 7.5 is required for the efficient absorption of DNA to the membrane. 100 µl of isopranolol was added to the sample and mixed to increase the yield of DNA fragments < 500bp and > 4 kb. The sample was then placed into a QIAquick spin column inside a 2 ml collection tube and centrifuged for 1 min at 17,900 × g (13,000 rpm). The flow-through was discarded. 600 µl of wash buffer PE was added and centrifuged for 1 min at 17,900 × g (13,000 rpm) with the flow-through discarded before further centrifuging for 1 min at 17,900 × g (13,000 rpm). QIAquick spin column was then placed in a new eppendorf and 37 µl of elution buffer was added and allowed to stand for 1 min before centrifuging for 1 min at 17,900 × g (13,000 rpm). The DNA was then stored at -20ºC.

2.3.5.2. DNA sequencing reaction

BigDye is a set of dye terminators labelled with high sensitivity dyes (401). PCR products and plasmids were sequenced using dideoxynucleotide chain termination. The PCR-based sequencing contained 5 µl of PCR product as template, dideoxynucleotides (ddNTPs), each base of which is labelled with a different fluorochrome (A-Dye terminator labelled with dichloro [R6G] - green, C-Dye terminator labelled with dichloro [ROX] - blue, G-Dye terminator labelled with dichloro [R110] - yellow, T-Dye terminator labelled with dichloro [TAMARA] - red), deoxynucleotides (dATP, dCTP, dGTP, dUTP), DNA Taq polymerase, buffer and 1 µl (1.6 pmol) of one primer.

During PCR amplification the DNA is replicated in the 5'-3' direction from the double stranded template created by the primer catalysed by AmpliTaq DNA polymerase incorporating the dNTPs. The ddNTPs lack a 3' hydroxyl group, required for the formation of 3'-5' phosphodiester bond and so the chain extension is halted when a ddNTP is incorporated into the product. This results in the product being labelled with a single fluorochrome-labelled ddNTP. The final result is a mixture of DNA molecules of different lengths each ending in a labelled ddNTP. The cycling conditions used were 96ºC for 10 secs followed by 50ºC for 5 secs and finally 60ºC for 4 mins before being repeated for 25 cycles.

2.3.5.3. Ethanol sodium acetate precipatation

The aim of precipitation is to isolate the sequencing reaction from the unincorporated dye terminators, which would interfere with the sequencing analysing. 2 µl of 3 M pH 5 sodium acetate and 50 µl of 100% ethanol was added to each extension product. The whole solution was then pipetted into a 1.5 ml microcentrifuge tube and centrifuged at 17,900 × g (13,000 rpm) for 40 mins at 4ºC. This precipitates the extension products out of solution and pellets them. The supernatant containing the unincorporated dye terminators was then discarded. The pellet was rinsed with 150 µl of 75% ethanol and centrifuged at 17,900 × g (13,000 rpm) for 5 mins at 4ºC. The ethanol was discarded before further centrifugation at 17,900 × g (13,000 rpm) for 2 mins at 4ºC to remove the last of the ethanol. The remaining ethanol was removed by careful pipetting followed by drying on a heat block. The pellet was resuspended in 10 µl of formamide by vortexing and then collected by brief centrifuging. The product was then ready for loading onto the sequencer.

2.3.5.4. DNA sequencing

The extension products were placed into a 96 well plate and loaded onto a ABI3130xl DNA sequencer. The products with the nucleotide sequence indicated by the terminating ddNTP were separated on high resolution polyacrylamide gel electrophoresis (PAGE) and read by a laser detecting the differing wavelengths of the dyes. DNA sequences were confirmed using MacVector™.

2.3.5.5. Cloning of polymerase chain reaction products

Sometimes it was necessary to clone a PCR product in a plasmid vector prior to sequencing. The cloning vector required has essential features including independent replication within a host cell, antibiotic resistance to aid future selection and multiple cloning sites to allow insertion of the foreign DNA.

PCR products were cloned by insertion into a plasmid vector using the pGEM-T® kit. Taq polymerase has template-independent terminal transferase activity which adds dATP to the 3' end of PCR products. PCR products can then be ligated into a linearised pGEM-T vector, which has complementary single 3’ T overhangs. These single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by Taq polymerase.

There are two methods by which the vector allows selection of bacteria that has been transfected with a vector that carries an insert. Firstly bacteria that are not transformed are killed by ampicillin in the medium as the vector carries a gene for ampicillin resistance. Secondly when plated onto 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) agar plates, bacteria containing the religated vector lacking an

insert form blue colonies as the transformed cells contain a functional gene, LacZ, that converts the colourless substrate X-gal into a blue coloured precipitate. If a PCR product is successfully ligated into the cloning site of the vector, the LacZ gene is disrupted so no active enzyme can be produced and the colonies remain white. These colonies were then picked with a sterile pipette and grown before plasmid extraction.

2.3.5.5.1. Ligation

The DNA from the PCR product and vector were joined together in the following mixture ready for transformation into the bacteria. 1 µl T4 DNA ligase, 5 µl of buffer, 3 µl of PCR product and 1 µl of vector, mixed and left for 1 hour.

2.3.5.5.2. Transformation

JM109 strain of E.coli was used as the host organism. 5 µl of the ligation reaction was mixed with 200 µl of bacteria by gently stiring and then left on ice to rest. The cells were then heat shocked in a water bath at 45ºC for 45 secs to allow the vector to enter the cell and then put on ice for 2 mins.

300 µl of lysogeny broth (LB) medium was added followed by their incubation at 37º in a shaking incubator (200 rpm) for 1 hour. Warm LB agar plates, supplemented with ampicillin, were prepared by spreading 100 µl of 0.1 M isopropyl thiogalactoside (IPTG) and 20 µl of Xgal and dried for one hour. The transformed cells were then spread on the plates and dried before being inverted and incubated at 37ºC overnight. A control plate without bacteria and one with bacteria without vector were included to ensure no contamination.

2.3.5.5.3. Picking colonies and culture

The following day the plates were placed in the fridge (4ºC) till the afternoon to aid colour development. White colonies were then picked for culturing. Each colony was placed in 2 ml of LB containing ampicillin (50 µg/ml) and cultured overnight on a shaking incubator (300rpm). A control without a colony was included.

2.3.5.5.4. Purification and extraction of plasmid DNA.

Plasmid DNA was extracted from culture using a Qiagen Miniprep kit. The kit uses an alkaline lysis method to disrupt the bacteria's cell walls. The proteins and bacterial DNA are precipitated in the centrifuge and removed. The plasmid DNA remains in the supernatant and is applied to a silica column which absorbs it and, after serial washing with ethanol, is eluted ready for sequencing.

1.5 mls of cells in culture was centrifuged at 10,000 × g (8,000 rpm) for 2 mins to pellet the cells and remove the medium. The cells were resuspended by vortexing with the addition of 250 µl of buffer P1. 250 µl of buffer P2 was added to lyse the cells and tubes were inverted to mix the solutions 4-6 times. 350 µl of buffer N3 was added

immediately and the tubes mixed by inversion to precipitate the bacterial DNA. The solution was then centrifuged to pellet the bacterial DNA at 17,000 × g (13,000 rpm) for 10 mins and the supernatant containing the plasmid DNA was pipetted into the Uprep tubes. The supernatant was then centrifuged at 17,000 × g (13,000 rpm) for 1 min to allow the plasmid DNA to be absorbed by the silica membrane. The membrane was then washed with 650 µl of buffer PE and centrifuged at 17,000 × g (13,000 rpm) for 1 min with the flow through discarded before a further 1 min of centrifugation at 17,000 × g (13,000 rpm). The column was then placed in a new eppendorf and the plasmid DNA eluted in 50 µl of elution buffer by centrifuging at 17,000 × g (13,000 rpm) for 1 min.

2.3.5.5.5. DNA sequencing of DNA plasmids

T7 and SP6 promoter primers were used to sequence the plasmid DNA following similar methods outlined previously (Section 2.3.6.2-4).

2.3.6. Immunohistochemistry of human tissue sections and fixed cells

Immunohistochemistry is the in situ detection of antigens by labelled antibodies to estimate protein expression in tissue. The antigen displays one or more specific binding sites or epitopes that can be identified by using labelled antibodies. This antigen-antibody reaction can be visualised through fluorescent dyes, enzymes or radioactive markers. Immunohistochemistry was first described in 1950 using fluorescent dyes (402) and subsequently techniques have included enzyme labels such as horseradish peroxidase (403). To increase the amplitude of staining, the chromogen can be indirectly linked to the primary antibody via an avidin-biotin complex (ABC) method (404).

2.3.6.1. Tissue preparation

Following either endoscopic or surgical removal, the tissue was fixed in 10% w/v neutral buffered formaldehyde (NBF) for 24-48 hours. Underfixation of tissue as well as delay in using cut sections has been shown to lead to loss of antigenicity (405). The samples were then cut for clinical purposes before being archived at room temperature. 4 µm thick sections were cut (by a histopathology technician for the TMAs) and mounted on positively charged slides to improve tissue adherence (Superfrost Plus) close to the date of staining.

To deparaffinise the sections the slides were placed in Xylene and rehydrated through serial bathing in graded alcohol solutions.

2.3.6.2. ABC method

The ABC method is a three layer indirect method. The unlabelled antibody binds to the antigen. A secondary antibody which is biotinylated, expressing multiple molecules of biotin, binds to the first antibody. Next a complex of avidin, streptavidin, bound to biotin horseradish peroxidase complex, is added which can bind to each of the biotin labelled

sites on the secondary antibody. This increases the number of sites available for chromogen binding compared to a direct method.

Finally to visualise the location of the primary antibody-secondary antibody-streptavidin-biotin-horesradish peroxidase complex, the chromogen 3-diaminobenzidine (DAB) is used. DAB is oxidised by horseradish peroxidase to produce a brown substrate and signifies the antigen expression that can be visualised down the microscope.

To counterstain the background, blue Mayers haematoxylin was applied before the sections were dehydrated through graded alcohols, cleared of alcohol in xylene and mounted with plasticiser and xylene (DPX) resin under a coverslip.

2.3.6.3. Blocking to prevent non-specific binding

To prevent unwanted binding a number of blocking steps were undertaken. The majority of tissues have endogenous peroxidase that oxidase the chromogen DAB as well as endogenous avidin binding activity. Both can lead to non-specific binding. To prevent endogenous peroxidase activity 0.5 % hydrogen peroxidase in methanol was added prior to the antigen retrieval step. To prevent endogenous avidin binding to the tissue avidin is added to saturate its binding capacity. Then the tissue is incubated with excess biotin. All biotin binding sites are then filled, preventing non-specific binding as biotin can only bind avidin once (406).

2.3.6.4. Antigen retrieval

Chemically fixing tissue with formaldehyde and heating to 60 ºC to embed the tissue can lead to the denaturement of the antigenic epitopes. Antigen retrieval techniques aim to treat the tissue by the application of solutions and heat to help antigens regain their immunogenicity. However, the mechanism for this remains unclear (407). Ethylene diamine tetracetic acid (EDTA) (pH 8.0) or citrate buffer (pH 6.0) were used for antigen retrieval using a Tecnolec Superwave 800 microwave to bring the buffer to the boil.

2.3.6.5. Titration and controls

Commercially available antibodies that had been previously used in formalin fixed paraffin embedded (FFPE) tissue were used. The optimal dilution of an antibody provides the most intense specific staining with as little background staining as possible. The optimal dilution can be affected by many factors including tissue fixation technique, antigen retrieval method, antibody batch and age of tissue (408). To ascertain the optimal dilution, multiple sections known to be positive to the antibody were stained at various dilutions to determine the most accurate staining (Table 2-12).

A negative control section was included in each run. This had all reagents and all steps apart from the addition of the primary antibody. In place of the primary antibody a

non-immune serum was used. All staining of the test tissue could then be confirmed as specific staining after comparison with the negative control slide.

Table 2-12: Antibody, antigen retrieval and dilutions used in immunohistochemistry

Antibody	Dilution	Retrieval method	2ry antibody	Supplier
PASD1	1:150	ER2:20, HIER pH6	Rabbit	Sigma
SPAG9	1:100	ER2:20, HIER pH6	Rabbit	Sigma
NY-ESO-1	5µg/ml	ER2:20, HIER pH6	Mouse	Santa Cruz
MAGEC1	1:50-1:100	ER2:20, HIER pH6	Rabbit	Abcam
HORMAD1	1:800	ER2:20, HIER pH6	Rabbit	Sigma
MAGEA9	1:50	ER2:20, HIER pH6	Rabbit	Abcam
CTAGE1	1:50-1:500	ER2:20, HIER pH6	Rat	Santa Cruz
MAGEA3	10µg/ml	ER2:20, HIER pH6	Rabbit	Lifespan
CD3	1:2000	ER2:20, HIER pH6	Mouse	Leica
CD4	1:80	ER2:20, HIER pH6	Mouse	Leica
CD8	1:50	ER2:20, HIER pH6	Mouse	Leica
Foxp3	1:20	ER2:20; HIER pH6	Mouse	eBioscience

2.3.6.6. Manual immunohistochemistry protocol

1. Deparaffinise sections in xylene, 2 × 5 mins, and rehydrate through graded alcohols (5 mins each) to 70%.
2. Inhibit endogenous peroxidase with 0.5% hydrogen peroxidase in methanol (10 mins).
3. Wash in TRIS/HCl buffered saline, pH 7.6 (TBS) 3 × 2 mins.
4. Perform antigen retrieval procedure for primary antibodies.
5. Wash in TBS 3 × 2 mins.
6. Drain slides and apply avidin solution, 20 mins.
7. Rinse TBS 3 × 2 mins.
8. Drain slides and apply biotin solution, 20 mins.
9. Rinse TBS for 3 × 2 mins.
10. Drain slide, apply culture medium, 20 mins.
11. Drain slides and apply primary antibodies at appropriate dilutions, incubate overnight at 4ºC at remove for 30 mins the following morning before continuing.
12. Wash TBS for 3 × 5 mins.
13. Drain slides and apply biotinylated second stage antibodies at appropriate dilutions, 30 mins.
14. Wash TBS for 3 × 5 mins.
15. Drain slides and apply streptavidin biotin-peroxidase complexes at appropriate dilution, 30 mins.
16. Wash TBS for 3 × 5 mins.
17. Drain slides and apply DAB substrate, for 5 mins.
18. Rinse in TBS.
19. Wash in running water.
20. Counterstain with Mayers haematoxylin.

21. Blue sections in running tap water.
22. Mount slides by dehydrating through graded alcohols, clear in Xylene and mount in DPX.
23. Allow to dry for 24 hours prior to image capture.

2.3.6.7. Automated immunohistochemistry protocol

The method used differed slightly from the manual protocol and were performed at University Hospital Southampton Foundation Trust (UHSFT). Automated immunostaining (Ventana XT, Ventana, Tucson, AZ, USA) was performed in a CPA-accredited clinical cellular pathology department using antibodies optimised to national diagnostic standards (NEQAS).

2.3.6.8. Image capture

All sections were digitally image captured to enable sections to be viewed and scored using OlyVIA 2.4 software.

2.3.6.9. Scoring of immunohistochemistry

The scoring of the tissue sections was performed under the supervision of Professor Gareth Thomas, Professor of Pathology.

2.3.6.9.1. Semi-quantitative

Within each core only tumour cells or representative areas of normal oesophagus or BO were scored. The incorporation of 3 cores on the TMAs for each patient and area of interest ensured representative areas were available for scoring.

It has been shown that semi quantitative scoring by a human observer is as accurate as quantitative scoring (409, 410). Scoring of CTAs was done in four levels: negative 0%; low <25%; moderate ≥25-50% and high ≥50%.

The intensity of staining was scored if it varied between sections using a similar four point scale: 0 for no staining; 1 = mild; 2 = moderate and 3 = strong.

Following scoring, all expression data needed grouping to facilitate statistical analysis. This was done using justifiable cut-offs (high-low/positive-negative/either side of the median) or quartiles.

2.3.6.9.2. Quantitative

The majority of published work studying immune infiltration of tumours used quantitative scoring. 3 high powered fields (×20 magnification) with the highest density of the marker of interest was scored, with a mean taken, providing a total scoring area of 0.1944 mm^2. This is in keeping with previous studies assessing the number of intra-tumoral immune infiltration (411).

Training of the observers was undertaken on a series of OACs from the TMA. A total of 25 randomly selected patients from the total cohort were used to assess the

intraobserver and interobserver variation in the TIL scoring. Two observers (F.N. and L. MM.) assessed the inflammatory cell infiltrate independently and without knowledge of clinicopathological information. The interobservers' intraclass correlation coefficient was >0.6 and were considered acceptable in keeping with previous studies. F.N. scored all slides, and these data were used in the analysis (412).

2.3.6.10. Tissue microarray

The construction of the TMAs required the identification of suitable cases from a prospective clinical database of oesophagogastric resections maintained by the cancer nurse specialist Donna Sharland. The original pathology report was then acquired and subsequently pseudoannoymised. This allowed the H&E slides to be located in the pathological archives of UHSFT. The slides were then assessed by Professor of Pathology, Gareth Thomas, to identify representative tissue areas to be included in the TMA. Corresponding blocks were then retrieved from the pathology archives of UHSFT and the relevant H&E section laid on top of the block to enable marking at the appropriate point (Figure 2-1).

Tissue cores, three per representative tissue area, were then physically extracted from the blocks (donor blocks) and placed into the TMA (recipient block) using the MiniCore® 3 (Aphelys Minicore 2, Mitogen, Harpenden, UK). A meticulous record of the positioning of the donor tissue was produced by the transfer of the pseudoaanoymised clinical data into the TMA designer® 3 software (Alphelys, France).

Orientation spots were included to allow configuration of the TMA. Normal tissue was included on the TMA to act as internal controls.

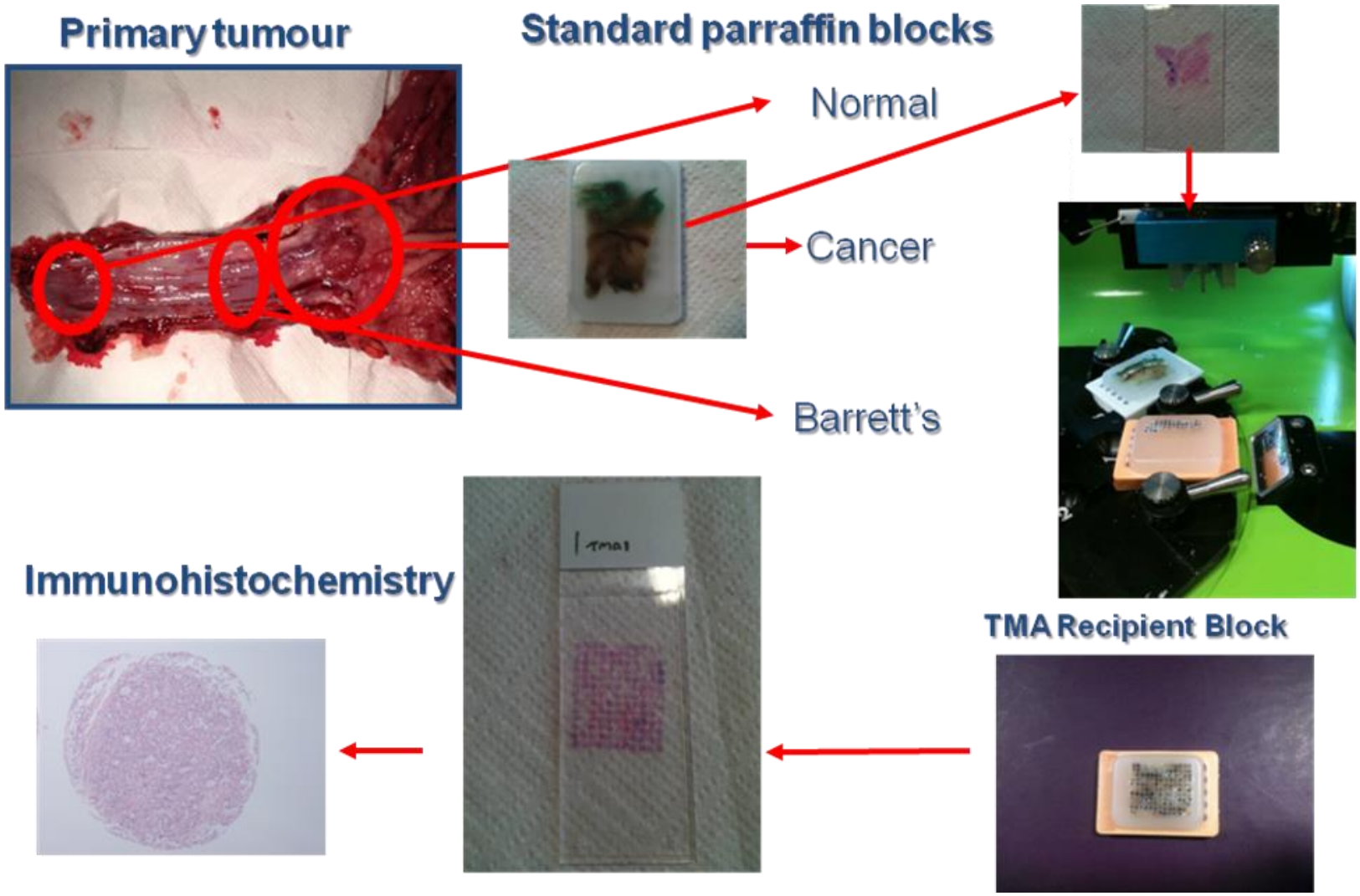


Figure 2-2: Steps involved in the construction of a tissue microarray

2.3.7. Immunogenicity of selected cancer testis antigens

To assess antigen specific T-cells and their response, a tetramer assay and an enzyme-linked immunosorbent spot (ELISPOT) assay were performed. The tetramer assay is a method to quantify T cells that recognise specific antigenic peptides (413). For this particular assay it relies on the TCR of CD8+ T cells recognising a nine amino acid peptide derived from the antigen of interest bound to a HLA class I molecule. To enable the T cell response to be measured peptides were loaded onto fluorescently labelled tetramers of HLA class I molecules. The fluorescent tetramers bind to T cells that express TCR against the specific peptide presented by the specific HLA molecule and are detected by flow cytometry (FACs).

FACs uses light to classify cells based on their differences in light scatter and fluorescence. When light hits the cells, it is scattered in either a forward or sideways direction. Forward scatter (FSC) is related to the size of the cell, whilst side scatter (SSC) depends on the granular content of the cell. Although each individual cell will be different FSC and SSC will be similar between cell types and so cell types of interest can be selected, gated, to allow further analysis on this gated population. The use of multiple fluorochrome labelled antibodies allows for specific groups with multiple attributes to be assessed like tetramer positive CD8+ cells.

The ELISPOT assay provides quantification of cytokines secreted by T cells that recognise a specific antigen. PBMCs were incubated with a specific antigen in IFN-Ɣ antibody coated wells. T cells capable of recognising the specific antigen will secrete the cytokine of interest and a secondary antibody to IFN-Ɣ is used to detect where IFN-Ɣ has bound to the IFN-Ɣ antibody coating the plate. A biotinylated detection antibody, a streptavidin enzyme conjugate, was then added. To visualise the amount of IFN-Ɣ secreted, a substrate is added that forms an insoluble precipitate when catalysed by the enzyme, showing as a dark spot. Due to the low level of T cells responding to some TAA a cultured ELISPOT was performed to expand the population of T cells that are immunogenic. This was done by culturing the number of cells in vitro for 14 days in the presence of IL-2 and the antigen of interest so as to increase the frequency of immunogenic T cells and the resulting secretion of IFN-Ɣ.

2.3.7.1. Human leucocyte antigen A*02 typing

Human leucocyte antigen A*02 (HLA-A*02) typing of patients was performed as specific peptides are loaded onto specific HLA molecules and presented to specific TCR of T cells. HLA-A*02 was chosen as it has a high frequency of expression and a significant number of vaccines utilise this HLA molecule to induce an immunological response. The tetramer assay was therefore designed using peptides that could be loaded onto HLA-A*02 molecules.

1×10^6 cells were centrifuged at 1,500 rpm for 5 mins and resuspended in FACS buffer. 100 µl was added to one FACS tube and stained with 2 µl of isotype control with 900 µl stained with 50 µl of HLA-A*02 and placed in the dark for 15 mins. The cells were then centrifuged at 1,500 rpm for 5 mins and washed with 1 ml of FACS buffer and centrifuged for a further 5 mins at 1,500 rpm. The cells were then resuspended in 200 µl of FACS buffer ready for FACS. The isotype control was used to ensure the specificity of the primary antibody.

2.3.7.2. Peptide epitope design

TAA are presented on the cell as individual peptides bound to HLA class I molecules. Recognition of the HLA-peptide complex by the TCR of CTLs triggers the tumour cell's death. The aim of vaccines delivering particular epitopes that are recognised by particular HLA class I molecules, is to enhance this immune response. A significant limit to antigen recognition appears to be the failure to incorporate peptides satisfactorily on the HLA molecule (414).

Computational methods have therefore been developed to predict HLA binding affinity of potential peptides so as to reduce the need to perform numerous binding assays. The prediction models identify optimal epitopes based on known optimal binding features between particular epitopes and HLA molecules. These features include anchor sites which are areas on the HLA molecule that incorporate certain peptide sequences more readily and greater focus on the N- and C-termini of the peptide sequence due to its higher specificity compared to the middle of the peptide (415, 416). Based on published literature and the use two of these prediction models, SYRPETHI and BioInformatics and molecular analysis section (BIMAS), suitable peptide epitopes were manufactured by peptide synthetics for MAGEA3 and CTAGE1 (417, 418). A PASD1 epitope was previously designed and available in Dr Sahota's laboratory with a tetramer made by Leon Douglas and Dr Patrick Duriez from the in house protein core facility.

2.3.7.3. Human leucocyte antigen A*02 tetramer assay

Tetramers were made by Leon Douglas and Dr Patrick Duriez from the in house protein core facility. PBMCs were stained with calcein to establish a live cell population and then stained with the tetramer before finally being stained with surface antibodies (CD3, CD4 and CD8) to assess live cell's synchronous tetramer and T cell stained populations. Controls including unstained, surface antibodies only and an irrelavent tetramer (HIV or EBV) with surface antibodies were included.

PBMCs were thawed and resuspended in warmed medium and counted using tryphan blue (Section 2.3.2). PBMCs were then washed in 1 ml of PBS twice and centrifuged at 1,500 rpm for 5 mins between washes before being resuspended in 1 ml of PBS and 2 µl of calcein and left in the dark for 30 mins. The cells were then washed in 1 ml of PBS and 1% w/v of BSA twice and centrifuged at 1,500 rpm for 5 mins between

washes and split to give appropriate tubes for staining: unstained (1 $\times 10^5$ cells); surface antibodies only (5 $\times 10^5$ – 1 $\times 10^6$ cells); surface antibodies and test CTA tetramer (1 $\times 10^6$ cells) and surface antibodies and irrelevant tetramer (5 $\times 10^5$ – 1 $\times 10^6$ cells). Tetramers were diluted to 5 µg/ml in 50% w/v FCS and 50% w/v FACS buffer and the solution centrifuged at 13,000 rpm for 5 mins just before use to pellet the aggregates. The relevant PBMCs were then incubated in 50 µl of the appropriate tetramer for 30 mins in the dark. PBMCs stained with tetramer were then washed in 150 µl of FACS buffer and centrifuged at 1,500 rpm for 5 mins before being resuspended. Appropriate tubes were stained with 10 µl of CD3-APC-Cy7, 20 µl of CD4-FITC and 2.5 µl of CD8-PerCP-Cy5.5 for a further 20 mins in the dark. Cells were washed in 1 ml of FACS buffer and centrifuged at 1,500 rpm for 5 mins before being resuspended in 200 µl of FACS buffer ready for acquisition on the FACS canto II.

Data analysis was performed using FACS diva software allowing suitable gating to assess live, singlet, CD8+ and tetramer positive cells with controls to ensure no background or non-specific binding was being assessed inappropriately.

2.3.7.4. Enzyme-linked immunosorbent spot assay

To assess peptide specific IFN-Ɣ secretion from PBMCs ELISPOT, assays were performed on a 96 well Elispot plate with each condition repeated in triplicate. In a class II laminar flow hood the wells were primed by adding 15 µl of 35% w/v ethanol and left for 1 min before being washed with 200 µl of filtered PBS per well 3 times. The wells were then coated with 100 µl of anti-human IFN-Ɣ (50 µg/ml) diluted in PBS and left overnight.

The excess of anti-human IFN-Ɣ was then removed and the wells washed with sterile PBS 3 times. Appropriate solutions were added to wells in triplicate to include negative controls: media only; HIV peptide 20 µg/ml and positive controls: viral peptide pool 20 µg/ml; phytohemagglutin PHA 10 µg/ml; and the test peptide at 20 µg/ml. Peptides were diluted in complete RPMI (sodium pyruvate, penicillin, streptomycin, glutamine and 10% w/v of AB serum) media. The plates were incubated at 37ºC, 5% CO_2 for 1 hour. PBMCs were then thawed in complete RPMI, counted, diluted and resuspended to allow 100 µl of cells to be added to each well to give 4 × 10^5 cells per well. The exception was the PHA wells which had 1 × 10^5 cells per well due to its high affinity for PBMCs. The cells were incubated in their individual conditions for 16 hours at 37ºC with 5% CO_2.

The next day the cells were removed and the plates washed with PBS and 0.1% w/v of tween using 150 µl per well 5 times before 100 µl of the biotinylated detection antibody diluted in sterile PBS and 1% BSA was added to each well. This was left for 90 mins at 37ºC before washing again with PBS and 0.1% w/v of tween using 150 µl per well 6 times before 100 µl streptavidin alkaline phosphatase diluted in sterile PBS and 1% BSA was added to each well. This was left for a further hour at 37ºC before being washed with PBS and 0.1% w/v of tween using 150 µl per well 6 times.

Finally 100 µl of substrate, a zymed kit, was added and the plate was allow to stand for 9 mins or until spot development was evident. At this point the plate was emptied and was run under the tap to stop the reaction before being dried ready for the spots to be counted using the ELISPOT reader.

Analysis was performed taking the average from the three wells processed in the same conditions. A result was deemed positive if the test peptide wells had > 10 spots per well and a statistically significant greater number of spots than the negative control and exceeded the sample minus the background (cells only) (419).

2.3.7.5. Expansion of antigen specific CD8+ cells

To increase antigen specific T cell responses PBMCs were cultured with the test peptide. Controls included culturing in the presence of a viral peptide pool, positive control, and a non-specific peptide to act as a negative control.

On day one of expansion 1 ml of X vivo 15 media with 5% w/v of human AB serum containing 2×10^6 PBMCs were added to individual wells of a 6-12 well tissue culture plate. A further ml of media was added containing 20 µg of appropriate peptide to provide wells containing test peptide as well as controls. The cells were then incubated at 37ºC with 5% CO_2.

On day four and seven the 1 ml of media from individual wells was removed and replenished with media containing 40 IU of recombinant human interleukin 2 (rH IL-2) and the cells placed back in the incubator.

On day nine the cells were harvested, counted and resuspended in complete RPMI ready for ELISPOT assay or tetramer assay as before (Sections 2.3.7.3-4)

2.3.8. Immunological Antibody assay

A novel immunological assay developed by Serametrix Corporation was used to detect the presence of antibodies to a panel of tumour associated antigens including cancer testis antigens.

2.3.9. Databases

A prospective database had been maintained by Donna Sharland, cancer nurse specialist, for all upper gastrointestinal resections carried out at UHSFT and Spire Southampton since 2005. This was for the purposes of service evaluation and for supplying data for the National Oesophagogastric Audit. Rudimentary data existed on patients and it served as a starting point and was complete in identifying all resections during this time period.

The database was originally populated in Excel and underwent multiple transformations until being populated in statistical package for the social sciences (SPSS) software in a pseudoanoymised fashion so analysis could be performed. The database was secured on an encrypted server with suitable back up facilities to ensure data was not

lost. Additional data to be collected was decided after discussion with both clinical and scientific groups to ensure completeness. Validated scoring systems were chosen for data fields and TNM 7 was used uniformly to pathologically stage the patient's cancers. This required restaging tumours previously staged using TNM 5 or 6 (FN and TJU). The cohort of 2005-2010 was chosen as this represented a modern series of oesophagogastric cancer patients and incorporated uniform preoperative staging, neoadjuvant treatments and surgical treatment so as to avoid the Will Rogers phenomenon (420).

The consistency of the data was checked in a number of ways. Within data fields, clinicopathological characteristics, mean, median, range and outlying data. The validity of the clinical data was checked by analysing its ability to produce known associations between clinicopathological characteristics and survival. At certain times data was checked for its validity by the collection of data retrospectively by a third party and checking with data from the database (TJU, NC, RS and DS).

2.3.10. Statistical analysis

The majority of statistical analysis was performed using SPSS with PRISM, Excel and confidence interval analysis (CIA) being used occasionally (Table 2-3).

2.3.10.1. *P- values*

The null hypothesis is that no difference exists between groups with the alternate hypothesis being that a difference exists between groups. When the null hypothesis is rejected when a true difference does not exist, then a false positive has occurred, a type I error. The *p-value* is the statistical chance of a false positive occurring. If a *p-value* = 0.05, this means that the chance of a difference being a false positive is 1 in 20. If the *p-value* is very small there is a low probability that accepting the result will represent a type I error. The level of chance of a false positive below which one will reject the null hypothesis is the alpha value and by convention is 0.05. This is not the same as the difference between groups being large. It reflects the probability of the difference having occurred by chance being low. If 20 independent hypotheses are tested on the same data, with an alpha value of 0.05, one false positive association will occur. The probability of accepting the null hypothesis when the alternative is true represents a Type II error. If a large number of hypotheses are to be tested on a dataset then the *p-value* is sometimes adjusted to a lower level. Methods include the Bonferroni adjustment although this is not without criticism including the increase in type II errors (421).

2.3.10.2. Analysis of associations with clinicopathological data

Descriptive statistics were initially explored to assess differences in clinicopathological characteristics between groups. The majority of data did not have a normal distribution so the data was represented as medians with dispersion displayed as

ranges. Dependent on the number of groups being analysed, a variable Mann-Whitney U or Chi-square test (2 independent groups) and Kruskal Wallis test (3 independent groups) were used. For related samples from two time points the Wilcoxon signed test was used.

Logistic regression was used to provide multivariate analysis of associations of variables with an outcome. It is used to predict the probability of occurrence of an event by fitting data to a logistic function.

2.3.10.3. Kaplan-Meier univariate survival analysis

Ideally no patients would be lost to follow up and the statistical analysis would occur when all patients had died of the disease or of other causes. However to mitigate the impossible the Kaplan-Meier method allows meaningful survival analysis with incomplete data.

Patient survival times are ordered from smallest to largest in life tables from which the proportion of patients at risk of dying is calculated at regular time points. Patients who are lost to follow up between intervals are removed, or censored, from subsequent analyses. The resulting cumulative survival is plotted against time as a curve with censored cases being denoted by a cross.

It is often useful to produce a numbers at risk table so one can deduce the number of patients who are being used to calculate the proportion who are at risk of dying. The curves can be truncated at a suitable timepoint based on the number of patients involved or the length of follow-up.

The Log rank statistic is used to determine whether there is a statistical difference in survival between groups. This generates a total number of expected and observed deaths within groups using the life tables. This is then used to generate the log rank statistic, the *p-value* for which is derived from referring to a chi-squared distribution table.

2.3.10.4. Cox proportional hazards model – multivariate survival analysis

Cox proportional hazards model, often called Cox's regression, allows multiple variables to be simultaneously assessed to reveal information regarding size and independence of a relationship between variables.

The model generates a baseline survival curve and calculates the hazard ratio (HR) for a variable by measuring the effect of removing it and adding it to the model. Variables included in multivariate analysis routinely include variables that have significance on univariate analysis, as this suggests the ability of the variable to predict outcome remains constant over time. The size and independence of each variable's individual influence on overall survival is determined. A HR of <1 means that the variable, or increase in the variable, will predict an improved prognosis. A HR of >1 means that the variable, or increase in the variable, will predict a worse prognosis.

2.3.10.5. Receiver operating curve analysis

A receiver operating curve (ROC) is a graphical plot of the sensitivity, or true positive rate, against false positive rate, or 1-specificity or true negative rate of a binary variable. It provides analysis to determine a cut-off of a particular test to provide optimal sensitivity and specificity.

The area under the curve (AUC) is a measure of the diagnostic accuracy of a test. It quantifies the probability that the test will correctly classify those with and without the outcome being measured (422).

2.3.10.6. Power calculations

Power calculations were undertaken in order to determine the size of effects that the TMA would be able to detect. From previous published TMA work using other tissue types it was estimated that a sample size of between 100-250 cases would be necessary to look for the relatively small effects that molecular and immunological markers exert on prognosis. The study population was relatively fixed and the findings from previous published studies confirmed that approximately 100-200 cases would be suitable for analysis. We were also able to deduce from previously published work the routinely used scoring methods used for markers. The majority allocated the cases into high or low expression groups. The number of cases varied between groups depending on the immunological marker studied.

To confirm these assumptions a range of scenarios were investigated for between 100 and 250 patients. The alpha value was 0.05 and the power to detect an effect was set at 0.80. 100 or 200 patients would be required to observe a 15% or 10% difference between groups. The power calculations gave us reassurance and an idea of sizes of effect that could be found with differing results.

3. Chapter 3: Impact of integrated PET/CT in the staging of oesophageal cancer: a UK population-based cohort study

3.1. Introduction

The incidence of oesophageal carcinoma has dramatically increased over the last two decades (423, 424) although the prognosis for oesophageal cancer remains poor with overall 5-year survival rate of 7%. Oesophagectomy is generally recognised as offering the best prospect of long term cure, but is a major procedure with perioperative mortality in modern series of 2-10% and significant morbidity in up to 60% of patients (105). Post-operative quality of life is significantly compromised for 3-6 months and never returns in those who develop early post-operative recurrence or metastatic disease (425). Poor long-term survival for patients who appear to have complete tumour resection appears to be, in part, due to a failure to detect distant metastases at or before the time of surgery (104). Accurate pre-operative staging is thus essential in providing informed treatment choices for these patients.

Conventional imaging used for staging oesophageal cancer in the UK includes contrast enhanced CT of the chest and abdomen with positive or negative oral contrast load, and EUS, techniques that provide high quality anatomic information. EUS enables accurate assessment of the depth of invasion (T stage) together with accurate information regarding local nodal involvement (N stage). Recently pre-operative imaging has variably included the use of PET/CT. PET detects metabolically active tissue based on metabolism of glucose (101). Studies have reported that metastases missed at CT were subsequently identified using PET in more than 10% of patients with oesophageal cancer (426-428). Combined PET/CT imaging facilitates the separation of normal physiologic uptake from pathologic uptake, and enables accurate anatomical localization of functional abnormalities. PET/CT combines both multisection CT and PET capabilities in two sequential scans, avoiding the need for patient motion between the CT and PET components of the study, thereby leading to more accurate co-registration of the CT and PET data (102). The use of PET/CT is likely to add accuracy over PET alone by enabling morphological and metabolic information to be gained in one sitting allowing more precise interpretation. Use of PET/CT in routine clinical practice in the UK has been variable and dependent largely on availability of this imaging modality.

The aim of this study was to establish the effect of combined PET/CT on the management of a prospective cohort of newly diagnosed patients with oesophageal cancer across five cancer networks in England.

3.2. Materials and methods

A multicentre prospective study was undertaken of patients with newly diagnosed oesophageal cancer who underwent PET/CT scan. This study was supported and sponsored by the upper GI tumour panel of the South West Cancer Intelligence Service (SWCIS), a cancer registry encompassing a population of 6.6 million, across 5 cancer networks covering the south and south west of England. The study included patients from 12 NHS trustswith data collection coordinated by SWCIS. Ethical approval was not required for this study as this was an audit of patients undergoing PET/CT. One of the remits of SWCIS is to conduct audits to improve services to cancer patients and this audit was within this remit.

3.2.1. Patients

Patients with histologically confirmed oesophageal cancer either confined to the oesophagus or involving the oesophagogastric junction diagnosed between 1st October 2006 and 30th September 2007 were included. All patients included in this study were, at the time of their PET/CT, candidates for potentially curative treatment of their tumours. This included a small number of patients with disease staged as potentially inoperable by CT and EUS, but where the multidisciplinary team (MDT) still thought that surgery may be an appropriate management plan.

3.2.2. Imaging

All CT scans were undertaken on current generation multidetector CT machines and with thin-section acquisitions as optimized according to local practice. An oral load of positive or negative (water) contrast medium was administered prior to CT scanning and images obtained during the administration of intravenous contrast (volume generally 100-150 ml dictated by local practice). Images were viewed according to local practice and the final local radiological report was taken to be definitive.

EUS, where possible, was performed according to locally agreed protocols and the operator's report used as an end-point. PET/CT was obtained at a variety of sites according to local arrangements with both fixed site machines and mobile units being utilized. All studies were integrated PET/CT without intravenous contrast medium for the CT examination. Although there may have been some minor variation in local practice, PET/CT was generally undertaken after a 6 h fast. A standard dose of 400 MBq of 2-[18F]-fluoro-2-deoxy-D-glucose (FDG) was administered and imaging commenced after a 45 min uptake period. Blood glucose of 10 mmol/l was considered acceptable. Results were recorded at local MDT meetings.

PET/CT examinations were reported as positiveif there were areas of non-physiological uptake of tracer away from the primary tumour, suggesting the possibility of

metastasis and negative if there were no areas of positive uptake outside the tumour (no metastases).

All preoperative imaging was completed within a 4-week time period.

3.2.3. Patient management

The CT, and EUS findings, together with all other preoperative staging investigations, were reviewed and correlated at 12 local MDT meetings involving upper gastrointestinal surgeons, thoracic surgeons, medical oncologists, pathologist, radiation oncologists, and radiologists with PET/CT training. The tumour node-metastasis classification (fifth edition) proposed by the International Union Against Cancer was used for staging (429).

3.2.4. Data

Data was prospectively collected by means of proforma to include demographics, pre operative staging with and without PET/CT, histological staging, PET/CT baseline practice and effect on MDT management decision. Data were entered on to an Excel (TM Microsoft) spreadsheet and analysed.

3.3. Results

A total of 191 (149 male, 42 female) patients of median age 66 years underwent PET/CT (Table 3-1).

Table 3-1: Demographic and pathological characteristic of patients undergoing PET/CT

Demographic	No. of patients (n=191)
Age Group	
36-54	23 (12%)
55-64	61 (32%)
65-74	69 (36%)
75+	38 (20%)
Histology	
Adenocarcinoma	133 (70%)
Squamous Cell Carcinoma	49 (26%)
Other	9 (4%)
Site	
Upper	3 (2%)
Middle	26 (13%)
Middle/Lower	11 (6%)
Lower	105 (55%)
Junctional	46 (24%)

The findings of the PET/CT examinations are summarized in figure 3-1 indicating the distribution of PET/CT examinations that were positive and negative. True-positive results were those that identified metastases and synchronous pathology not detected by CT and

EUS, in contrast to false-positive results that were later shown to have incorrectly suggested the presence of metastases not detected by CT and EUS.

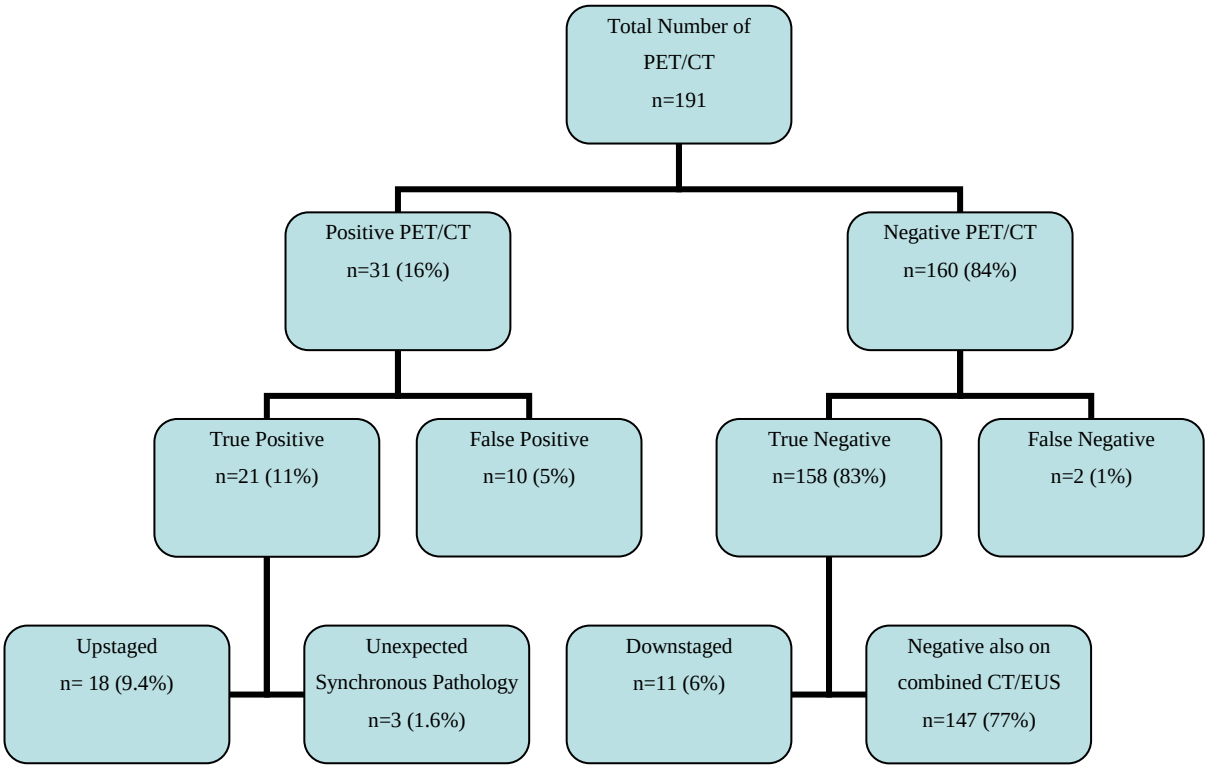


Figure 3-1: Chart illustrating the results of PET/CT for distant metastases.

Thirty-one (16%) patients had positive examinations consistent with distant metastases. Eighteen (9.4%) patients were subsequently upstaged and had distant metastases identified, including extensive lymph node involvement (n = 11), bone (n= 7), liver (n= 3), cervical lymph node (n= 1), and lung (n= 1). Preoperative CT did not involve the neck in 92 (48%) patients, but did include the neck in the one case where PET/CT identified an additional cervical node metastasis. The patients whose cancers were upstaged after PET/CT were initially staged with combined CT and EUS as IIa in three (6%) patients, III in 13 (13%) patients, and IV in two (20%) patients (Table 3-2).

Table 3-2: Distribution of integrated positron-emission tomography and computed tomography (PET/CT) positive results for distant metastases by conventional staging with CT and EUS

Stage CT and EUS	No. Cases (n (%))	Distant Metastases on PET/CT (n (%))
I	9 (5)	0
IIa	52 (27)	3 (6)
IIb	20 (11)	0
III	100 (52)	13 (13)
IV	10 (5)	2 (20)

The patients with true-positive results were confirmed by biopsy (n=11), and by subsequent clinical course, and further imaging (n=10). Three (1.6%) patients had unexpected synchronous pathology discovered during PET/CT. These lesions were a colonic adenoma, primary lung neoplasm, and carcinoid tumour of the lung.

The 10 (5%) patients with false-positive PET/CT results were from increased uptake in the liver (n= 4), kidney (n= 1), adrenal (n= 1), bone (n= 1), colon (n= 1), thyroid (n= 1), and mesentery (n= 1). These were confirmed as negative by appropriate negative investigations shown in table 3-3.

Table 3-3: Investigations confirming false positive integrated positron-emission tomography and computed tomography (PET/CT) results

Investigation Confirming false positive PET/CT Scans	Anatomical Site
Fine Needle Aspiration (FNA)	Thyroid n=1
Magnetic Resonance Imaging (MRI)	Bone n=1; Liver n=1
Biopsy	Kidney n=1; Adrenal n=1
Colonoscopy	Colon n=1
Laparoscopy	Liver n=4

Negative PET/CT was reported in 160 (84%) patients with 158 (83%) patients having true-negative results with eight (4%) being down-staged in the MDT meeting. Coeliac/M1a node involvement was excluded at PET/CT in seven patients and liver metastases were excluded in one patient. These findings were confirmed at operation and histologically in five cases, but three did not proceed to resection. Two patients decided not to proceed with surgery and one who tolerated neo-adjuvant chemotherapy poorly was subsequentlydeemed unfit for surgery.

The other 150 (79%) that were negative were also negative for distant metastases on combined CT and EUS. The primary tumour was not detected by PET/CT in 10 patients, seven (70%) of these cases were staged as T1 or T2 by combined CT and EUS. Two (1%) patients had false-negative PET/CT for distant metastases. In these cases conventional preoperative imaging was also negative for distant metastases. One patient was found to have metastasis in the peritoneum at operation, and the other in a neck lymph node, which became evident at preoperative clinical examination. Both false negative PET/CT results were positive for primary tumour uptake and were classified as T3 by CT and EUS. These two false-negative cases were both reviewed by the MDT and by an independent radiologist retrospectively and no change was made to the initial report of the PET/CT.

PET/CT was found to be helpful in planning management in 174 cases (91%), changed staging in 65 cases (34%), and management in 50 cases (26%). distant metastases was 91% and its specificity was 94%. The pathological stage at resection is summarized in table 3-4.

Table 3-4: Pathological stage post resection for patients who had negative PET/CT for metastatic disease

Pathological Stage post resection	Number of patients
0	4
I	14
IIa	32
IIb	11
III	49
IV	0
Unresected	61

CT was performed in every patient and EUS was performed in 132 patients (69%). PET/CT was performed routinely in 167 (87%) and selectively in 24 cases (13%) to assist with preoperative staging. Amongst those having PET/CT selectively, nine out of the 24 (38%) cases were shown to be truly positive for metastatic disease compared with nine out of 167 (5%) for those carried out routinely. The wait for PET/CT was reported to be less than 2 weeks in 10 out of the 12 centres in this study.

One hundred and seventy-three patients were deemed eligible for potentially curative resection after preoperative staging including PET/CT. One hundred and ten patients went on to have curative resection after two further patients were found to have distant metastases (false negative). PET/CT was positive for primary tumour uptake for all 110 patients that went on to have resection. The four patients subsequently shown to have stage 0 disease received neo-adjuvant chemoradiotherapy. Sixty-one patients did not go on to have oesophageal resection because of patient choice (n= 10), fitness for surgery post-neo-adjuvant chemoradiotherapy (n= 11), tumour progression (n= 18), death (n =2), and unknown (n= 20).

3.4. Discussion

This is the first UK multicentre study to review the impact of PET/CT amongst patients thought on the basis of CT/EUS to be candidates for curative treatment based on CT/EUS. PET/CT suggested distant metastases in 16% of patients and these metastases were confirmed in 9%. This is consistent with previous single centre series where 10% or more of patients have been found to have previously unsuspected metastatic disease in oesophageal cancer after PET/CT (430-432). As well as contributing to pre-treatment staging, unexpected synchronous pathology such as colonic adenoma, primary lung neoplasm, and carcinoid, was found at PET/CT in a small number of cases, as previously reported in other series (432-434). It is, thus, important to critically analyse the results of PET/CT for distant metastases before they are accepted. This study found a false positive rate of 5% (n=10) and false negative rate of 1% (n=2). The findings of synchronous disease and false-positive PET/CT scans highlight the importance of precise

determination of the cause of areas of uptake before they are attributed to metastatic disease, in order to avoid denying potentially curative treatment to patients who do not have metastatic disease. Other authors have highlighted this. Taira *et al (435)* evaluated PET/CT with regard to the identification of bone metastases in a variety of malignancies, and found very high positive predictive values (PPV, 98%) when both PET and CT portions of the examination were in concordance. However, PET and CT examinations appear to be discordant relatively frequently, and the PPV is then reduced significantly (PPV, 61% for PET versus 17% for CT; negative predictive value, 83% for PET versus 39% for CT). Furthermore, in patients with solitary bone lesions for which the PET and CT findings are discordant, the PPV for integrated PET/CT is particularly low at 43% suggesting that this finding should be interpretated with great care to avoid denying potentially curative treatment (435). Recent review has found PET/CT to be unhelpful in differentiating *(1)* inflammatory changes and neoplastic processes in lymph node stations or lymphatic tissues (Waldeyer ring or appendix); *(2)* residual tumour and post-therapy changes immediately after surgery or radiation therapy; *(3)* benign thyroid adenoma and thyroid cancer; *(4)* focal physiologic bowel uptake and large or small bowel malignancies; or *(5)* focal physiologic uptake in the uterus during menstruation and uterine cancer (436). This has important implications on radiology workload in terms of validating areas of non physiological uptake on PET/CT by other imaging techniques or biopsy. With more experience and recognition of common patterns of spread, the need for confirmatory biopsy may be reduced when positive PET/CT findings are considered typical of metastatic disease.

It has been suggested that PET/CT had limited additional value and that it should be used selectively in advanced tumours (437-439). In one institution based study only 6.6% of stage III-IV oesophageal cancer had previously undetected metastatic disease (437). This multicentre study found metastatic disease in 13% of stage III/IV disease by conventional staging, as compared 6% of patients with stage IIa oesophageal cancer. PET/CT was used selectively in 13% (n=24) of cases possibly introducing a small selection bias towards greater identification of undetected distant metastases in more advanced stage disease by conventional imaging. A strength of this study is that it reflects the impact of PET/CT in current clinical practice, within which there is often a degree of uncertainty. PET/CT is used mainly in stage II/III disease and where doubt exists after conventional imaging, in those thought to have stage IV disease. Where doubt arose following conventional imaging of stage IV disease, eight out of the 10 were subsequently shown to be PET/CT negative for distant metastases, with two out of the 10 showing further metastases not already detected. The role of PET/CT is thus confirmed in advanced disease, although the available data are not yet sufficiently robust to define the role of PET/CT in early-stage disease. A recent review in this journal discussed further potential for PET/CT in assessing the effect of neoadjuvant therapy, and potentially in

determining the need for adjuvant treatment. Further applications that require investigation include its role as a prognostic marker for patient outcome and as an aid to intensity modulated radiation therapy (440).

Quality control of images and their interpretation is an important issue in a cancer registry population-based study and a potential weakness of such a study is the lack of quality control with regards to the interpretation of images. Thus this study includes results from a heterogeneous group of operators and radiologists with different levels of experience. However, it reflects current ongoing clinical practice. The PET-CT centres used for imaging patients were also part of the National Health Service (NHS) southern sector national contract and so subject to independent audit of reporting. The population included in this study is managed according to improving outcomes guidance with review of cases taking place in specialist centres according to network agreed protocols, which provides a good level of quality assurance. That the findings are similar to other single centre studies also provides a good degree of assurance that quality control was appropriate.

Imaging the neck as part of routine CT with or without ultrasound examination has been suggested to increase sensitivity for detection of metastatic disease (441) and the neck was not routinely scanned in 50% of patients in this study. The only patient with a solitary neck node in this study found on PET/CT had previously had his neck scanned at CT, which failed to diagnose this metastasis. Although there is good evidence for routine staging CT to include the neck, the present data do not suggest that this should substitute for PET/CT imaging.

MDTs themselves found PET/CT imaging to be helpful in management of patients with management changes occurring in 50 (26%) patients. The complex nature of patients' clinical course of treatment is evident by the fact that of 191 patients who were found to have disease potentially amenable to curative treatment, only 110 patients eventually went on to have resection. In this cohort of patients PET/CT has provided valuable true-negative data scans for over 80% of patients, with this finding apparently validated by pathological stage at resection and patients' early subsequent progress. The most important role of PET/CT potentially lies in reducing the chance of early recurrence post resection. This role is not yet confirmed and it is essential to carefully follow up this and other cohorts of patients having had PET/CT, in order to define the impact of PET/CT on early recurrence of disease.

4. Chapter 4: A prospective comparison of totally minimally invasive versus open Ivor Lewis oesophagectomy

4.1. Introduction

Surgical resection is the primary curative treatment for oesophageal cancer. The recent appearance of adenocarcinoma of the distal third and gastro-oesophageal junction as the predominant oesophageal tumour type in developed countries has led many groups to advocate a two stage surgical approach with intrathoracic anastomosis as the operation of choice for these tumours (21, 118, 119, 147). Advocates suggest that a combined trans-abdominal and trans-thoracic approach offers the optimum strategy for tumour clearance and two-field lymphadenectomy (115-117). The additional gastric resection required for adequate tumour clearance of junctional tumours may reduce the length of gastric conduit that would otherwise be required to reach the neck (118, 119). This is reflected in the 2010 UK National Oesophago-Gastic Cancer audit, where nearly three quarters of operations were performed by the Ivor Lewis (IVL) approach (120).

Despite the enthusiasm for a two stage approach in the open setting the uptake of minimally invasive two stage oesophagectomy (MIO-2) has been slow (128). The goal of minimally invasive surgical techniques is to reproduce the open operation in a format that reduces surgical trauma and improve patient outcomes. This is especially pertinent to oesophageal cancer operations where surgery in two body cavities is associated with significant morbidity. The first hybrid MIO was reported by Cuschieri nearly twenty years ago (129). Initial experiences raised concerns over safety with respiratory complications reported in as many as 59% of cases (130). Minimally invasive skills and equipment have subsequently advanced and MIO has re-emerged in a variety of forms. Recent systematic reviews and meta-analyses have concluded that MIO may reduce morbidity, hospital stay and is at least comparable to open resection for peri-operative outcomes (115, 128). More recently MIO has been associated with a rapid restoration of health-related quality of life (131). Up to 30% of oesophageal resections in the UK are presently conducted using minimally invasive techniques, but only a small proportion have reported thoracoscopic oesophageal mobilisation (120). The majority of published series report the outcomes of hybrid type or three stage MIO (115, 117, 128, 132-140), with few series of MIO-2 in the literature (115, 118, 119, 141-146).

Here, we report a consecutive series of MIO-2 compared with a consecutive series of open Ivor Lewis oesophagectomy.

4.2. Methods

A prospectively collected database of consecutive patients diagnosed with oesophagogastric cancer or high-grade dysplasia and treated with a two stage oesophagectomy in University Hospital Southampton Foundation Trust between 1st January 2005 and 12th November 2010 was reviewed. All patients were discussed at a specialist multidisciplinary team meeting (MDT). Standard staging investigations including

high-resolution computed tomography, endoscopic ultrasonography, integrated fluorodeoxyglucose positron emission tomography/computed tomography (PET-CT) and staging laparoscopy were performed according to local protocols. Patients considered suitable for potential surgical resection with tumours staged as T2 N0 M0 or above were considered for neoadjuvant chemotherapy. Surgery was performed by three surgeons at Southampton University Hospital NHS trust (JPB, JJK and ISB). Patients were selected for MIO or open surgery (including type of open resection) based on a combination of surgeon preference and availability, and patient related factors including, previous upper abdominal surgery, unfavourable anatomy, body habitus and tumour characteristics. One surgeon only performed open operations (ISB, n=14). The indications for open procedures performed after the introduction of MIO-2 by the minimally invasive surgeons (JPB: 02/06/2008 and JJK: 02/06/2008) are detailed in table 4-1 (n=8). MIO-2 was introduced in accordance with recommendations from the Association of Upper Gastrointestinal Surgeons and the Association of Laparoscopic Surgeons for the introduction of MIO (442). Local clinical governance procedures were followed during the introduction of MIO and data was provided for the National Oesophago-Gastric Cancer audit (120).

Table 4-1: Indication for open surgery after the introduction of MIO-2

Indication
Previous gastric surgery and intrabdominal adhesions
Local invasion into diaphragmatic crura
Previous open fundoplication and intraabdominal adhesions
Intermittent claudication precluding modified Llyold-davies position
Distal oesophageal GIST invading distal pancreas and splenic hilium
Difficult airway assessed as unsafe for prone one lung ventilation
10cm T3/4 M1A tumour borderline for respectability
Surgeon preference-training

The first 53 consecutive patients who underwent MIO-2 were included in the analysis (2nd June 2008-12th November 2010) and comparison was made with 53 consecutive patients who underwent IVL oesophagectomy (1st January 2005-12th November 2010). During this time period 220 resections for oesophageal cancer or high-grade dysplasia were performed. The other operations performed were 43 MIO-3, 60 left thoracoabdominal and 11 trans-hiatal procedures.

All patients in the IVL group underwent laparotomy and thoracotomy and all patients in the MIO-2 group underwent laparoscopic gastric mobilisation, abdominal lymphadenectomy and intracorporeal construction of the gastric conduit, with thoracoscopic oesophageal mobilisation, thoracic lymphadenectomy and intrathoracic anastomosis. All operations were led by a specialist upper-GI surgeon (JPB, JJK and ISB). All MIO-2 operations were performed by one of two surgeons who had been trained on overseas fellowships (JPB - Princess Alexandra Hospital, Brisbane and JJK - Royal Adelaide Hospital, Adelaide). Minimally invasive surgery was introduced in a step-wise fashion beginning with hybrid abdominal procedures (n=20), moving through a three stage

minimally invasive approach (MIO-3) (n=23), all with thoracoscopic oesophageal mobilisation, before embarking on MIO-2.

Data recorded included: demographics, tumour characteristics, indication, type of resection, operative times (defined as knife to skin to wound closure), estimated blood loss (calculated from suction bottles and weighed swabs), intensive care unit (ICU) stay, overall hospital stay (defined as day of surgery until day of discharge), histopathologic analysis of the surgical specimen, and mortality. TNM-7 was used to report tumour stage after analysis of pathology reports (443). Pathological tumour clearance ("R"-status) was determined according the Royal College of Pathologists system. Postoperative complications were graded according to the Clavien-Dindo classification and were recorded for their entire inpatient stay (444). Clavien-Dindo grades I and II represent minor complications, whereas grades III and IV represent major complications (grade III require radiological, endoscopic or surgical intervention, grade IV indicates life-threatening complication requiring intensive care management) (444). An anastomotic leak (AL) was confirmed by radiology (contrast enhanced multidetector CT scan or water soluble contrast studies), endoscopy or during surgical exploration. All patients were cared for by a specialist oesophago-gastric team who applied the same peri-operative regime to all patients. A protocol driven postoperative pathway was not followed. All symptomatic strictures were confirmed by endoscopy and treated with dilatation as required. Patients were routinely followed-up for 5 years post surgery according to the following protocol: 2-4 weeks post-discharge, 3 monthly for 1 year, 6 monthly for 2 years and yearly thereafter. Patients were also seen on an as required basis if symptomatic. Recurrence of disease during follow-up was defined as the first site or sites of recurrence with radiological or pathological confirmation. Site of recurrence was defined as local: anastomosis or local lymph nodes, nodal: regional lymph nodes and distant: distant nodal or distant organ recurrence.

4.2.1. Surgical technique

Ivor Lewis Oesophagectomy is performed in a standard fashion as described previously (114). MIO-2 begins with the patient supine for the laparoscopic stage including gastric mobilisation with abdominal lymphadenectomy, gastric resection, and conduit formation. This is followed by thoracoscopic oesophageal mobilisation and mediastinal lymphadenectomy, including subcarinal and all paraoesophageal nodes, in the prone position with single lung ventilation. The thoracic duct was routinely resected en bloc with the surgical specimen and controlled with Ligaclips (Ethicon Inc., Somerville, NJ, USA) or Hem-o-lock clips (Teleflex medical, NC, USA). Occasionally positive pressure (< 5cmH_2O) is used at the beginning of the thoracic stage to aid collapse of the right lung. The oesophagus is transected proximally using an articulating, linear stapler (ENDOPATH® ETS 45 Articulating Linear Cutters, Ethicon Endo-Surgery, UK Johnson and Johnson Medical Limited). A 25mm Tilt-Top™ anvil (Covidien Surgical, Ireland) is

modified extra corporeally by removal of the spring and fixation of the anvil in the tilted position with a 2-0 polydioxanone (PDS) suture to allow per oral placement. The anvil is delivered into the oesophageal stump via attachment to a 19 F airway exchange catheter (Cook Ireland Ltd) with the 2-0 PDS suture. An oesophagostomy is made in the oesophageal stump to allow the delivery of the anvil and the airway exchange catheter. The PDS suture is then cut to enable extraction of the airway exchange catheter and delivery of the Tilt Top™ anvil in the horizontal position. To ensure incorporation of oesophageal mucosa in the anastomosis a purse string is placed around the rod of the anvil. The lower most thoracic port site is enlarged to 3 to 6 cm to allow removal of the specimen. A 25mm Premium Plus CEEA circular stapler (Covidien Surgical, Ireland) is then placed into the delivered gastric conduit, and reintroduced for the side-to-end anastomosis (side of gastric conduit to end of oesophagus) above the level of the azygos vein (the anvil is introduced transthoracically in the open Ivor Lewis operation). The redundant portion of the gastric conduit is trimmed using an articulating, linear stapler (ENDOPATH® ETS 45 Articulating Linear Cutters, Ethicon Endo-Surgery, UK Johnson and Johnson Medical Limited), and a 28F chest drain is placed by the anastomosis.

Post-operative care continued as standard with epidural analgesia used routinely unless contra-indicated or failed when patient-controlled analgesia was used. The specimen was sent whole for pathological analysis. These surgical techniques have been described in full elsewhere (114, 118, 119).

4.2.2. Statistical analysis

Mann-Whitney U and Pearson's χ^2 tests were used as appropriate. A p-value < 0.05 was considered significant. Overall and disease-free survival were analysed by the Kaplan–Meier method calculated from the date of operation until the date of death or date of recurrence respectively. Analysis was truncated at one third of the cohort at risk. Data on patients who had survived until the end of the observation period were censored at that time point. Statistical analysis was performed with SPSS® version 19 (SPSS, Chicago, Illinois, USA) and Confidence Interval Analysis (CIA)-TL version 2.2.0 (T Bryant, University of Southampton).

4.3. Results

4.3.1. Demographic and pathological parameters

Patients who underwent MIO-2 or IVL were comparable for age, gender, ASA score, BMI, smoking history, preoperative stage, neoadjuvant treatment and pathological tumour characteristics (Table 4-2). The median age at the time of surgery was 66 years with a range of 36 to 85 years. There was a male preponderance of 83% (88 male, 18 female) that was similar between the groups. In keeping with standard practice in the UK, 72% of patients received neoadjuvant chemotherapy (MIO-2: 76% vs. IVL: 68%). Final

pathology revealed adenocarcinoma in 95 patients, squamous cell carcinoma in 7 patients and 1 patient each with adenosquamous carcinoma, basaloid carcinoma, gastrointestinal stromal tumour and high-grade dysplasia.

Table 4-2: Patients and tumour demographics comparing MIO-2 versus IVL

Variable		IVL	MIO-2	*p*
Number of patients		53	53	
Median Age (years) *		64 (36-81)	66 (45-85)	0.275†
Sex Ratio (M:F)		45:8	43:10	0.605 χ2
Pre-op BMI *		26 (18-41)	26 (16-37)	0.359†
Pre-op Smoker		21 (64)	22 (54)	0.387 χ2
Performance Status				0.157†
	0	19 (36)	11 (21)	
	1	29 (55)	37 (70)	
	2	5 (9)	5 (9)	
ASA Grade				0.981†
	I	10 (19)	4 (8)	
	II	32 (60)	44 (83)	
	III	11 (21)	5 (9)	
Tumour Site				0.054 †
	Middle 1/3	2 (4)	2 (4)	
	Lower 1/3	19 (36)	27 (51)	
	OGJ-S1	11 (21)	7 (13)	
	OGJ-S2	18 (34)	10 (19)	
	OGJ-S3	3 (6)	7 (13)	
Pre-operative Stage T				0.769†
	0	0 (0)	2 (4)	
	1	1 (2)	0 (0)	
	2	15 (28)	9 (17)	
	3	33 (62)	42 (79)	
	4	4 (8)	0 (0)	
Pre-operative Stage N				0.353 χ2
	0	14 (26)	10 (19)	
	1	39 (74)	43 (81)	
Pre-operative Stage M				1 χ2
	M0	52 (98)	52 (98)	
	M1	1 (2)	1 (2)	
Neoadjuvant Chemotherapy		36 (68)	40 (76)	0.388 χ2
Adjuvant Chemotherapy		9 (17)	12 (23)	0.465 χ2
Adjuvant Radiotherapy		2 (4)	1 (2)	0.598 χ2

4.3.2. Surgical outcomes

In all patients reconstruction was achieved with gastric pull-up in an orthotopic position. Intestinal continuity was restored via a stapled anastomosis (MIO-2: 53 vs. IVL: 52) in all but one operation, in the IVL group, when a hand-sewn anastomosis was performed. There were 4 conversions to open in the MIO-2 group all during the thoracic stage of surgery. The indications for conversion were: adhesions, anvil deployment, and unsatisfactory purse-string and to confirm haemostasis. The median duration of operation was 270 min (range 120–480min, Table 4-3). There was a significant difference in operative time of 60 min between the groups (MIO-2: 300 min vs. IVL: 240 min, $p<0.0001$). Median total intraoperative blood loss was 325 ml (range 0-3000 ml) and there was significantly less blood loss in the MIO-2 group (MIO-2: 300 ml vs. IVL: 400 ml, $p=0.021$). This reduction in blood loss translated into fewer intraoperative blood transfusions in the MIO-2 group (MIO-2, 3 patients required 4 units vs. IVL, 3 patients

required 9 units) but this was not statistically significant (p=0.95). Surgery was performed for tumours of the gastro-oesophageal junction or oesophageal lower 1/3 in 96% of cases (Table 4-2). There was no difference between lymph node yield between the groups (median nodal yield MIO-2: 18 (range 7-52) vs. IVL: 19 (range 7-50), p=0.584, Table 4-4). The mean number of positive nodes did not differ between the groups (MIO-2: 2 vs. IVL: 2). During pre-operative staging a similar number of patients in each group were believed to have involved local lymph nodes (MIO-2: 81% vs. IVL: 74%), on subsequent pathological analysis the actual proportion of patients with involved lymph nodes was 46% in both groups. R0 resection (tumour >1mm from the resection margin) was achieved in 81 patients (MIO-2: 43 vs. IVL: 38, Table 4-4). We have used the Royal College of Pathologists criteria that define an involved circumferential resection margin (R1) as any case where tumour is found to be ≤ 1mm from the resection margin. Using the criteria of the College of American Pathologists the R1 resection rate falls to 11% and does not differ between groups (IVL: 13% vs. MIO-2: 9%). There were no R2 resections in this series and no involved longitudinal margins. A total of 7 patients who underwent an R1 resection were suitable and agreed to undergo adjuvant therapy (MIO-2: 3 patients, IVL: 5 patients).

4.3.3. In hospital outcomes

The median inpatient stay was 12 days (range 7-101) with no difference between groups (MIO-2: 12 days, range 7-91 vs. IVL: 12 days, range 7-101, 95% CI for the difference between medians: -3 to +1). There was one death in each group (MIO-2: post-operative day 80 and IVL: post-operative day 101) both from sepsis following anastomotic leak leading to respiratory compromise. The rate of anastomotic leak was not significantly different between the groups (MIO-2: 5 patients (9%), IVL: 2 patients (4%), p=0.241). Complications according to the Clavien-Dindo classification were reported in 70 patients (66%), the majority (67%) of these were minor complications (grade I or II). A total of 21 patients had a major complication (grade III or IV, Table 4-3). Specific complications are detailed in table 4-5. There was no statistically significant difference in the rate of major complications between the groups (MIO-2: 12 patients (23%) vs. IVL: 9 patients (17%), p=0.465).

Table 4-3: Operative and surgical outcome data comparing MIO-2 versus IVL

Variable		IVL	MIO-2	*p*
Number of patients		53	53	
Extra Procedures		2 (4)	0 (0)	0.361 χ2
Stapled Anastomosis		52 (98)	53 (100)	0.315χ2
Median duration of operation (min)		240 (120-	300 (180-	0.0001 †
Median blood loss (ml) *		400 (0-3000)	300 (0-1250)	0.021 †
Median total length of stay (days) *		12 (7-101)	12 (7-91)	0.358 †
Median ITU stay *		1 (0-34)	1 (0-58)	0.995 †
Conversions		n/a	4 (8)	n/a
Anastomotic Leak		2 (4)	5 (9)	0.241 χ2
Clavien Dindo Max grade				0.242 †
	0	21 (40)	15 (28)	
	1	2 (4)	1 (2)	
	2	20 (38)	24 (45)	
	3	2 (4)	6 (11)	
	4	7 (13)	6 (11)	
	5	1 (2)	1 (2)	
Return to Theatre		5 (9)	6 (11)	0.750 χ2
Stricture rate		18 (34)	10 (19)	0.078 χ2
Median dilatations		2 (1-3)	2 (1-5)	0.107 †

Mann Whitney U test † Pearsons χ2 test; Values in parentheses are percentages unless indicated.

*Values in parentheses are range

Table 4-4: Oncological outcome comparing MIO-2 versus IVL

Variable		IVL	MIO-2	*p*
Number of patients		53	53	
pT				0.820 †
	0	1 (2)	3 (6)	
	1	11 (21)	11 (21)	
	2	17 (32)	12 (23)	
	3	24 (45)	26 (49)	
	4	0 (0)	1 (2)	
pN				1 †
	0	29 (55)	29 (55)	
	1	13 (25)	13 (25)	
	2	8 (15)	8 (15)	
	3	3 (6)	3 (6)	
pM				0.169 χ2
	0	49 (93)	52 (98)	
	1	4 (8)	1 (2)	
Vascular Invasion		16 (30)	14 (26)	0.666 χ2
Perineural Invasion		9 (17)	5 (9)	0.251 χ2
Lymphatic Invasion		9 (17)	6 (11)	0.403 χ2
Median maximum tumour Size d		30 (0-110)	25 (0-80)	0.117 †
Morphology				0.437 †
	Ulcer	32 (60)	38 (72)	
	Polypoid	17 (32)	7 (13)	
	Fungating	1 (2)	1 (2)	
	Diffuse Infiltrating	3 (6)	7 (13)	
Tumour Type				0.380 †
	AC	48 (91)	47 (89)	
	SCC	3 (6)	4(8)	
	AS	0 (0)	1 (2)	
	Dysplasia	0 (0)	1 (2)	
	Basaloid	1 (2)	0 (0)	
	GIST	1 (2)	0 (0)	
Differentiation				0.913 †
	G1	5 (9)	7 (13)	
	G2	18 (34)	15 (28)	
	G3	28 (53)	30 (57)	
	G4	1 (2)	1 (2)	
	GX	1 (2)	0 (0)	
Associated Barrett's		18 (34)	19 (36)	0.839 χ2
Median Nodal Yield *		19 (7-50)	18 (7-52)	0.584 †
Resection Clearance				0.253 χ2
	R0	38 (72)	43 (81)	
	R1 (CRM)	15 (28)	10 (19)	
	R1 (Long)	0 (0)	0(0)	
Pattern of Recurrence	**Local**	1 (2)	2 (4)	0.558 χ2
	Nodal	8 (15)	5 (9)	0.374 χ2
	Cervical	3	1	
	Thoracic	4	3	
	Abdominal	1	0	
	Thoracic + abdominal	0	1	
	Distant	17 (32)	9 (17)	0.071 χ2

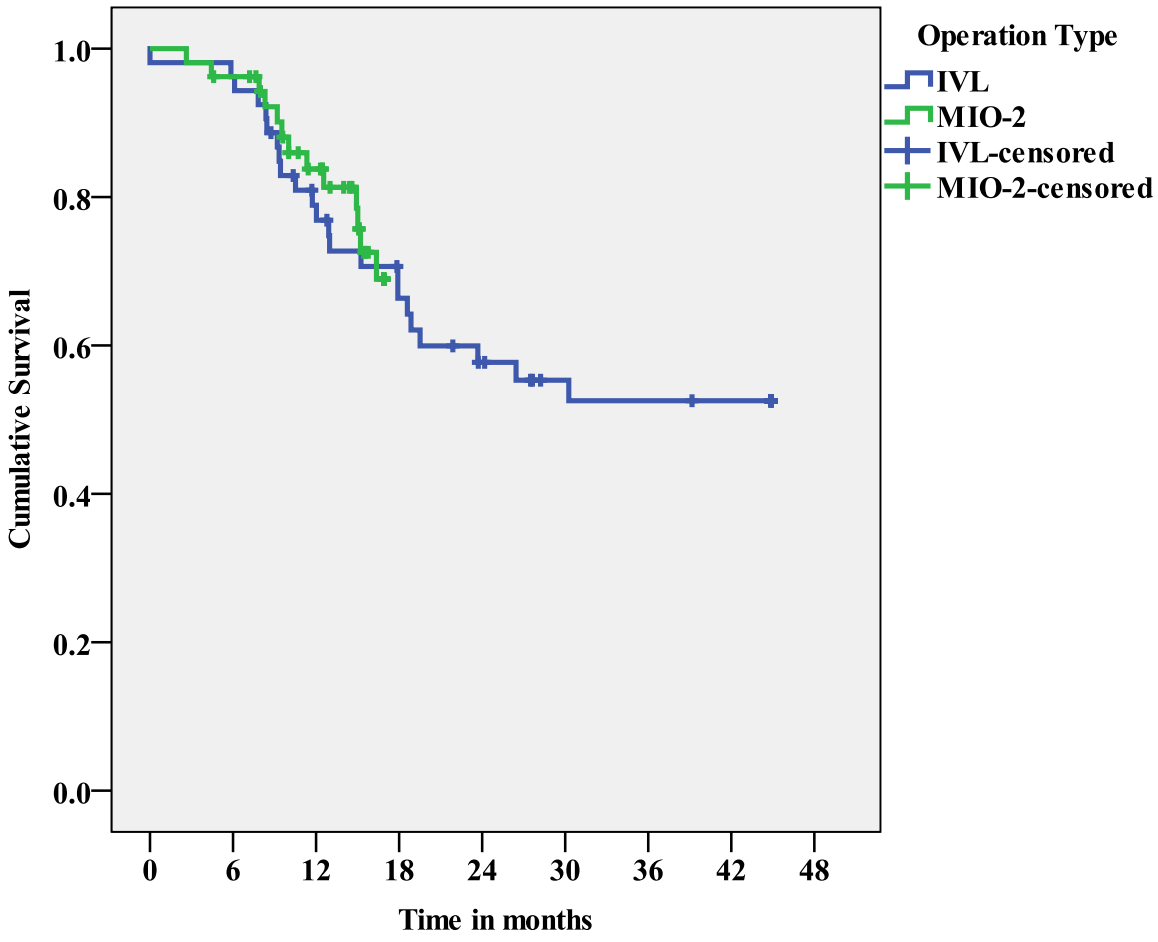
Table 4-5: Postoperative inpatient complications of minimally invasive compared to open Ivor Lewis oesophagectomy (2005-2010)

			IVL	MIO-2
Number of patients			53	53
Clavien Dindo grade				
Minor	**1**	**Atelectasis**	0	1
		Urinary retention	1	0
		Hypophosphataemia	1	0
	2	**Atrial fibrillation**	6	6
		NSTEMI	1	1
		Pulmonary complications	10	10
		Pulmonary embolism	1	0
		Chyle Leak	0	1
		Wound infection	1	1
		Others	1	5
Major	**3**	**Anastomotic leak**	0	2
		Pulmonary complications	0	4
		Chyle leak	1	0
		Diaphragmatic hernia	1	0
	4	**Anastomotic leak**	1	2
		Pulmonary complications	4	4
		Pulmonary embolism	2	0
	5 (death)	**Anastomotic Leak**	1	1

Values in parentheses are percentages. NSTEMI – Non ST segment elevated myocardial infarction.

4.3.4. Survival and medium term outcomes

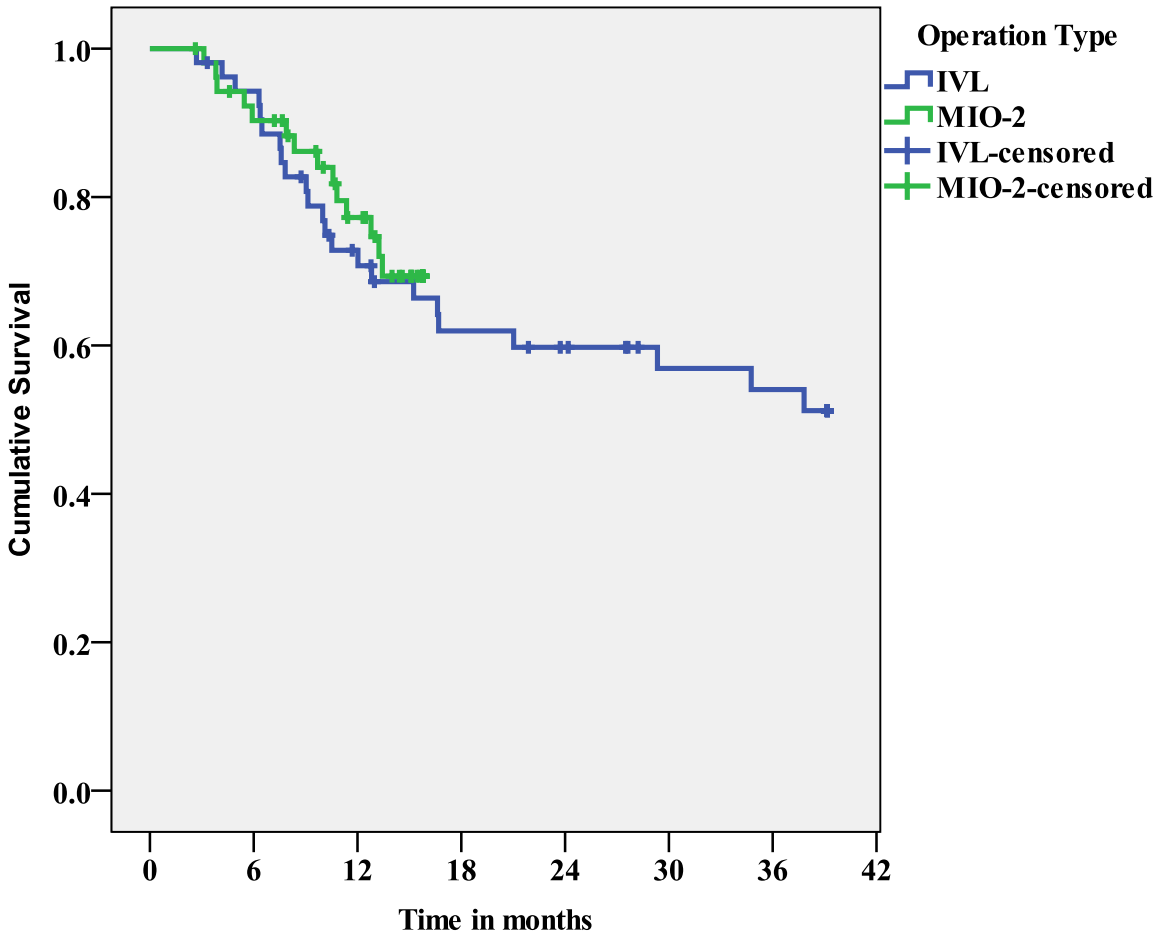
The median follow-up was 17 months (range 3 – 76 months, IVL: 24 months vs MIO-2: 14 months). There was no statistically significant difference between the groups in terms of overall survival (Figure 4-1) or disease free survival (Figure 4-2). A total of 39 patients (37%) died during follow-up (MIO-2: 15 vs. IVL: 24). Disease recurrence was observed in 40 patients (38 %) and there was no statistical difference between the groups. Anastomotic stricture requiring intervention was observed in 28 patients (26%) and there was no statistically significant difference between groups. These patients required a median of 2 (range 1-5) dilatations.



No. at risk

Months	0	6	12	18	24	30	36	42
IVL	53	51	39	31	25	20	19	18
MIO-2	53	50	37	17				

Figure 4-1: Overall survival comparing MIO-2 versus IVL (Log rank *p*=0.900)



No. at risk							
Months	0	6	12	18	24	30	36
IVL	53	49	35	28	25	20	19
MIO-2	53	46	33	15			

Figure 4-2: Disease free survival comparing MIO-2 versus IVL (Log rank *p*=0.675)

4.4. Discussion

In this series we have compared a continuous series of 53 MIO-2 with 53 IVL comparable for patient demographics, BMI, smoking history, performance status, tumour stage (pre-operative and pathological), and tumour site. The majority of resections were performed in patients with adenocarcinoma (96%) who had undergone neoadjuvant chemotherapy (76%) and was equal between groups. We have shown MIO-2 to be comparable with IVL in terms of peri-operative (major complications and anastomotic leak) and short term clinical outcomes.

The retrospective nature of this type of analysis is subject to shortcomings including selection bias and data collection. Patients were not randomized to MIO or open surgery and some baseline variables (known and unknown) may be linked to outcome e.g. BMI, previous surgery etc. However, in this study some of these shortcomings are mitigated by the fact that the data was vigorously recorded prospectively and the cohort included consecutive two stage oesophagectomies performed in a single centre by an

experienced surgical team. The two surgeons performing MIO-2 (JPB and JJK) had been previously trained on overseas fellowships and had performed over 50 hybrid or MIO-3 procedures, all with thoracoscopic oesophageal mobilisation, eliminating the learning curve from the analysis. In this series pre-operative T and N stages were similar between the groups and this was confirmed after pathological analysis; 49% of patients who underwent MIO-2 were found to have T3 disease compared to 45% in the IVL group. Similar proportions of patients received neoadjuvant chemotherapy (MIO-2: 40 patients (76%), IVL: 36 patients (68%)) with similar rates of significant tumour regression (tumour regression grade 1-3) as measured by the Mandard score(83) between groups (MIO-2: 15 patients (38%), IVL: 15 patients (42%)). This may explain the difference between perceived preoperative lymph node involvement (MIO-2: 81% vs. IVL: 74%), and actual lymph node involvement found in the pathological specimen (MIO-2: 45% vs. IVL: 45%) (445, 446). These data demonstrate that our series is a comparison between open and minimally invasive surgery for the stages of disease most commonly encountered in western practice.

The most recent meta-analysis of MIO and hybrid procedures versus open oesophagectomy suggests a benefit for MIO in terms of length of stay, overall morbidity and pulmonary complications (128). These findings are not observed in our series. However, MIO-2 is without disadvantage when compared directly with IVL for these parameters. The overall morbidity in this series appears high (66%). We have been careful to include all events that are outside normal postoperative recovery, many of which have no consequence for the patient. Only 21 patients (20%) had a major (grade III-IV) complication. Recent series have suggested a reduction in respiratory complications as a major benefit in favour of MIO (128). A reduction in respiratory complications from 57% to 29% in favour of MIO was used to calculate sample size for the multi-centre Traditional invasive versus Minimally invasive esophagectomy (TIME) trial (447). In our series of well matched patients, with comparable patient numbers to the TIME-trial, there was no statistically significant difference in major respiratory complications between groups (IVL: 5 vs. MIO-2: 10; p=0.164). We await the outcome of the TIME-trial with interest. Anastomotic leak occurred in two patients after IVL (managed conservatively n=0; managed with reoperation n=2) versus five patients after MIO-2 (managed conservatively n=2; managed with reoperation n=3) in this series. The only difference in anastomotic technique involved using a trans-oral technique for introducing the anvil in the MIO-2 group compared to transthoracic introduction in the IVL group. This introduces an additional staple line in the MIO-2 group but otherwise was identical to the IVL group and its relevance to this non-significant difference is unknown. The consequences of anastomotic leak are often considerable, and much larger series will be needed to determine whether this is a sustained pattern that becomes statistically significant with a larger sample size.

Much debate remains regarding the role of minimally invasive surgery and the adequacy of oncological resection. In this series MIO-2 was equivalent to IVL in terms of R0 resection and lymph node harvest. The R1 resection rate in this series seems relatively high (23.6%) but is below the proportion of patients found to have positive circumferential resection margins in the UK National Oesophago-gastric Audit 2010 (positive CRM 29%, positive LRM 6.4%)[8]. Some surgeons worry that the demanding nature of a thoracoscopic oesophageal mobilisation may limit the length of oesophagus removed above the tumour. It is important to note that in this series there were no cases of positive longitudinal margins and MIO was without disadvantage in this respect. Furthermore, the adequacy of circumferential clearance is in part dependent on the definition of circumferential margin involvement. Using the criteria of the College of American Pathologists the R1 resection rate is 11% in this series, with no significant difference between groups. In addition, overall survival and disease free survival are equivalent between the groups. The relatively short median follow-up in this series (IVL: 24 months vs MIO-2: 14 months) may mask a potential long-term difference and further follow-up is required. However it does serve to highlight patient selection, short and medium term outcomes that may be masked by just looking at surgical and in hospital perioperative outcomes.

The traditional reservations regarding minimally invasive surgery, including the management of intraoperative complications and the maintenance of orthodox oncological principles remain, and uptake of MIO lags behind the use of minimally invasive techniques in colorectal and liver surgery (448, 449). Furthermore, gastric conduit necrosis has been suggested as a complication specific to MIO (82). We observed no incidence of gastric tube necrosis in this series, in keeping with other large series of MIO-2 (450). This suggests that gastric tube necrosis may be a specific complication of the three-stage procedure.

A significant benefit for MIO is the faster return to pre-operative quality of life (131). The median life expectancy after oesophagectomy is relatively short and only 20-40% of patients can expect to be alive at 5 years. Therefore any gain in health-related quality of life after MIO is likely to have a significant impact on this patient population. It is unlikely that any real difference exists between open oesophagectomy and MIO in terms of morbidity, mortality and oncological efficiency. Therefore, quality of life related outcomes must be the focus of future studies. The heterogeneity of operations described in previous studies may mask the potential benefits of MIO in relation to quality of life. Our patients will only be in a position to make truly informed decisions about the benefits of MIO when it is compared with IVL, the standard procedure against which all other techniques are measured (147). A randomized trial comparing MIO-2 with open IVL oesophagectomy with quality of life related data as the primary end-point and cost-effectiveness as a secondary analysis is now required.

The findings of this single centre series support a pragmatic approach to oesophageal surgery for cancer. We believe that surgeons who perform open surgery with good results should feel comfortable to continue with open surgery until the true benefits of MIO have been established. Our data also supports the proponents of MIO. We have demonstrated that MIO is without detriment when compared directly with IVL for all measured parameters. MIO can be introduced safely within the context of a specialist team and enthusiasts should be encouraged to publish their results and contribute to future trials.

5. Chapter 5: Risk assessment using a novel score to predict anastomotic leak and major complications after oesophageal resection

5.1. Introduction

Cancer of the oesophagus is the most rapidly increasing cancer in the western world and is associated with a 5-year survival of less than 15% (21). Oesophagectomy is performed as the primary curative treatment for local and loco-regional oesophageal cancer (82).

Morbidity and mortality occur in significant numbers following oesophageal resection despite improvements in preoperative staging (150), patient selection (151), surgery (152) and perioperative care (153). Major complications including anastomotic leak (AL) are reported at rates of 3.8-30% (154, 155) with mortality rates varying between 0% and 11% in modern series (156, 157). The majority of patients who develop an AL or a major complication following oesophagogastrectomy are diagnosed when symptomatic or critically ill (158) and often require intensive care management and reoperation. Rapid diagnosis of AL is essential to limit contamination and minimise sepsis (158) as this may lead to improved clinical outcomes both in the short and long term. There is gathering evidence that septic complications, including AL, lead to reduced disease free and cancer specific survival in colorectal cancer (159) and initial reports show this may also be the case in oesophageal and gastric cancer (160-163).

Preoperative predictors of outcome have been extensively investigated for patients who undergo oesophageal resection. They provide important information for appropriate selection of patients for oesophagogastrectomy (105) and allow an informed decision by the surgeon and patient. In addition they will become increasingly useful, as institutions and surgeons are compared, by enabling risk adjustment for their case mix (165). Clinical characteristics reported to have associations with short term patient outcome include: age (166), presence of comorbidity (105, 167, 168), hospital volume (169) and the oesophagogastric physiological and operative severity score for the enumeration of mortality and morbidity (O-POSSUM) (451, 452). These indexes may be useful for stratification of patients into high or low risk groups, but in the post-operative period they tell us little about the development of complications on an individual basis.

The degree of peri-operative systemic inflammation has been shown to predict the development of postoperative complications and outcome after surgery involving cardiopulmonary bypass (453). Furthermore, in a study of 2,300 patients admitted to a surgical intensive care unit in New York, Talmor et al found that the magnitude of the pro-inflammatory response on the second intensive care unit day was a useful predictor of outcome in critical surgical illness (454). Oesophageal surgery is associated with a profound systemic inflammatory response that may predispose to, and be compounded by, the development of complications. Postoperative predictors of morbidity and mortality following oesophageal surgery are scarce with the majority predicting prognosis after a complication has occurred, or with a focus on mortality. Predictors include serum

albumin, organ dysfunction and the requirement for mechanical ventilation (156). The systemic inflammatory response syndrome (SIRS) may result in changes in heart rate, blood pressure and temperature but these changes tend to occur when the patient becomes symptomatic. A simple test that can predict the onset of complications before symptoms develop is required. Postoperative biochemical markers of the systemic inflammatory response are measured routinely following major cancer resection. To date, only a handful of studies have assessed their association with postoperative complications. One study found low albumin on the first post operative day after oesophagectomy to be associated with major complications but not AL (174). Other studies have largely focused on C-reactive protein (CRP) following colorectal surgery (175-178), with only two studies looking at oesophageal resections in small cohorts (179, 180).

The aim of this study was to assess the predictive accuracy of three commonly used systemic inflammatory biochemical markers (albumin, CRP, and white cell count (WCC)) for AL and major complications/death (Clavien-Dindo (CD) grade III-V) in a large cohort of oesophageal resections. In addition, we describe the development and prospective validation of a novel score combining albumin, CRP and WCC that has superior diagnostic accuracy than each marker independently.

5.2. Methods

5.2.1. Patients

For this retrospective study, a prospectively collected database of consecutive patients undergoing oesophagogastric resection with oesophageal anastomosis treated at a single UK university hospital between January 2005 and December 2011 was reviewed. This was divided into a development data set of 258 patients (January 2005 – December 2010) and a validation data set of 42 patients (January 2011 – December 2011). All patients followed a contemporary treatment pathway with description of general parameters included and anlaysed included in chapter 2 (Chapter 2, 2.2.3).

Data recorded included demographics, O-POSSUM, tumour characteristics, type of resection, estimated blood loss (calculated from suction bottles and weighed swabs), histopathologic analysis of the surgical specimen, postoperative clinical parameters, postoperative complications and mortality. TNM-7 was used to report tumour stage after analysis of pathology reports (393). Pathological tumour clearance (“R”-status) was determined according the Royal College of Pathologists system. Postoperative complications were graded according to the Clavien-Dindo (CD) classification (444). CD grades I and II represent minor complications, whereas grades III and IV represent major complications (grade III require radiological, endoscopic or surgical intervention, grade IV indicates life-threatening complication requiring intensive care management), and grade V indicates death. An AL was defined as a leak sufficient to cause symptoms and confirmed

by radiology (contrast enhanced multi-detector CT scan with on-table oral contrast or water soluble contrast studies), endoscopy or during surgical exploration. All patients were cared for by a specialist oesophagogastric team who applied a similar perioperative regime to all patients.

5.2.2. Factors analysed

Serial daily blood samples were taken for routine laboratory analysis of WCC, albumin and CRP in the preoperative and postoperative period (postoperative day (POD) 1-7). The WCC (reference range 4.0-11.0 $\times 10^9$/l) was analyzed with an automated haematological blood analyzer (Sysmex TS-500 (Sysmex UK Ltd)). Serum concentrations of albumin (normal range: 35-48 g/l) and CRP (normal range: 0-7.5 mg/l) were measured in an auto-analyzer UniCel DxC800 (Beckmann Coulter Inc)). The coefficient of variation for these methods, over the range of measurement, was less than 2% as established by routine quality control. Heart rate (HR), respiratory rate (RR) and temperature, ºC, was reviewed and the maximum value recorded for each POD.

5.2.3. Statistical analysis

Descriptive data are represented as median and range with Kruskal-Wallis, Mann Whitney U and Pearson's chi-squared tests used as appropriate for comparison. Univariate and multivariate binary logistic regression analyses were used to assess the relationship between serial postoperative blood tests (WCC, CRP, and albumin) and NUn score with major complications (including death) and AL as outcomes. A p value <0.05 was considered statistically significant for all tests. The diagnostic accuracy of WCC, CRP, albumin and NUn score were assessed through the use of receiver operator curves (ROC). The NUn score was developed using the log likelihood ratio test of variables after multivariate analysis with respect to AL on post-operative day (POD) 4 using a development data set of 187 cases (January 2005 – December 2010). All variables that showed significance on univariate analysis were entered into the final model. The NUn score was then tested on a prospective validation data set of 39 cases on POD 4 (January 2011 – December 2011). Statistical analysis was performed with SPSS® version 19 (SPSS, Chicago, Illinois, USA). Confidence intervals were produced using Confidence interval analysis (CIA)-TL version 2.2.0 (T Bryant, University of Southampton) at 95% or greater dependent on sample size.

5.3. Results

5.3.1. Development data set

5.3.1.1. Patient profile and complications

A total of 258 patients (202 men and 56 women) underwent oesophageal resection for presumed cancer or high grade dysplasia during the study interval. The median age

was 67 (range 37-85) years at the time of resection. There was no evidence of invasive disease on post-operative pathology in 24 patients (9.3%). One hundred and fifty-six (60.5%) patients had neo-adjuvant chemotherapy.

Major complications (CD III-V) were observed in 70 patients (27%). There were 7 (2.7%) in-patient deaths. 26 (10%) patients had an AL diagnosed at median post-operative day (POD) 7 (range: 5-15) (managed conservatively n=12; managed with reoperation n=14). Detailed patient demographics and O-POSSUM showing the lack of relationship with complications, using the CD classification, are presented in table 5-1.

5.3.1.2. Markers of the systemic inflammatory response

There was no relationship between preoperative levels of albumin, CRP, and WCC in patients having an uneventful postoperative course compared to those that developed a complication or AL (Table 2 and 3). Only 39% of patients had a preoperative CRP recorded. After uneventful oesophageal resection WCC peaked on POD 2 (median 11.15 $\times 10^9$/l (range 4.4-29.7 $\times 10^9$/l)) and CRP on POD 3 (median CRP 224 mg/l (range 5-500 mg/l)). Both markers declined thereafter. Albumin levels decreased from a median of 39 g/l (range: 22-45 g/l) preoperatively to median 24 (range: 16-32 g/l) on POD 1 and remained stable before rising from POD 6 (Table 5-2, Figure 5-1). Of note there was no statistically significant relationship between heart rate (HR), respiratory rate (RR) and temperature with complications or specifically AL over the first 7 PODs.

5.3.1.3. Predictive factors of CD III-V

In patients who developed major complications (CD III-V) serum albumin was statistically significantly lower than those patients who experienced no complications from POD 3 ($p<0.008$) until POD 7 ($p<0.0001$). Postoperative CRP was higher in those patients with major complications compared to those with no complications from POD 3 and this persisted to POD 7 but was only statistically significant for POD 5 and 7 ($p=0.005$ and 0.035 respectively). WCC peaked at median 11.55 $\times 10^9$/l (range 3.5-43 $\times 10^9$/l) on POD 2 and was persistently higher than those patients with no complications to POD 7 (Figure 5-1C). This difference reached statistical significance on POD 5 to 7 (Table 5-2, Figure 5-1).

5.3.1.4. Predictive factors of anastomotic leak

In patients who developed an AL, all systemic inflammatory markers showed statistical significance with a continued persistent reduction in albumin and persistent elevation of WCC and CRP compared to patients who did not develop an AL (Table 5-3, Figure 5-2). Clinical indicators of a SIRS response did not differ between groups (POD 4: median HR, no leak: 95 vs. AL 95 ($p>0.05$); median RR, no leak: 18 vs. AL 20 ($p>0.05$); median temperature, no leak: 37.2 vs. AL 37.5 ($p>0.05$)).

Table 5-1: Clincopathological characteristics of oesophagogastric resection patients grouped by complications using the Clavien-Dindo classification for the development data set 2005-2010

		No complication (CD 0, n=84)	Minor complication (CD 1-2, n=104)	Major complication (CD 3-5, n=70)	*P value*
Operation Age*		65 (37-82)	68 (51-85)	66 (45-82)	0.039 †
Sex ratio (M:F)		66 (79):18 (21)	80 (77):24 (23)	56 (80):14 (20)	0.887 χ2
Preoperative BMI*		26 (18-43)	25 (18-42)	25 (14-40)	0.864 †
Preoperative smoker		38 (35)	40 (36)	32 (29)	0.823 χ2
O-POSSUM		16 (12-35)	16 (12-30)	16 (12-39)	0.293 †
ASA	**1**	15 (18)	6 (6)	4 (6)	0.056 †
	2	59 (70)	83 (80)	54 (77)	
	3	10 (12)	15 (14)	12 (17)	
Tumour site	**Upper 1/3**	0 (0)	0 (0)	1 (1)	0.826 †
	Middle 1/3	5 (6)	6 (6)	6 (9)	
	Lower 1/3	32 (38)	42 (40)	22 (31)	
	OGJ-S1	9 (11)	19 (18)	10 (14)	
	OGJ-S2	14 (17)	10 (10)	10 (14)	
	OGJ-S3	15 (18)	18 (17)	16 (23)	
	OGJ - Unclassified	9 (11)	9 (9)	5 (7)	
pT	**T0**	7 (8)	9 (9)	8 (11)	0.941 †
	T1	18 (21)	21 (20)	16 (23)	
	T2	21 (25)	23 (22)	11 (16)	
	T3	35 (42)	47 (45)	30 (43)	
	T4	1 (1)	4 (4)	3 (4)	
	T IS/HGD	2 (2)	0 (0)	2 (3)	
pN	**N0**	40 (48)	56 (54)	42 (60)	0.175 †
	N1	16 (19)	23 (22)	14 (20)	
	N2	17 (20)	13 (13)	10 (14)	
	N3	11 (13)	12 (12)	4 (6)	
pM	**M0**	80 (95)	102 (98)	70 (100)	0.140 χ2
	M1	4 (5)	2 (2)	0 (0)	
Resection clearance (R0)		69 (82)	76 (73)	59 (84)	0.143 χ2
Vascular Invasion		25 (30)	33 (32)	22 (31)	0.995 χ2
Lymphatic Invasion		15 (18)	13 (13)	10 (14)	0.584 χ2
Perineural Invasion		12 (14)	10 (10)	12 (17)	0.332 χ2
Neoadjuvant chemotherapy		56 (67)	55 (53)	45 (64)	0.119 χ2
Surgical	**IVL**	37 (44)	50 (48)	25 (36)	0.547 †
approach	**Mckeown**	12 (14)	19 (18)	20 (29)	
	LTA	21 (25)	18 (17)	13 (19)	
	Transhiatal	14 (17)	17 (16)	12 (17)	
Blood loss (ml)*		300 (0-1440)	305 (0-3000)	350 (20-1700)	0.397 †
Operation time (mins)		240 (105-401)	240 (75-480)	242 (110-425)	0.560 †

Kruskal Wallis test † Pearsons χ2 test Values in parentheses are percentages unless indicated. *Values in parentheses are range

5.3.1.5. Development and assessment of a scoring system (NUn score)

Using binary logistic regression analysis, albumin, CRP and WCC were combined in a model formula (Figure 5-4) based on their relationship with AL on the first day all markers had statistical significance, postoperative day 4 (Table. 5-3 and 5-4).

The NUn score was then assessed for its relation to complications (major complication or death (CDIII-V) and AL) using a ROC curve. The NUn score on POD 4 was predictive of an anastomotic leak (NUn score >10: sensitivity 95%, specificity 49%, diagnostic accuracy 0.801 (95% confidence interval (CI) 0.692-0.909, $p<0.0001$)) and a major complication or death (NUn >10: sensitivity 69%, specificity 59%, diagnostic accuracy 0.723 (95% CI 0.627-0.818, $p<0.0001$)). The NUn score was compared to individual markers of the systemic inflammatory response (albumin, CRP, WCC) and showed greater diagnostic accuracy, sensitivity, specificity and statistical significance in predicting AL (Table 5-3, 5-4 and 5-5, Figure 5-2 and 5-3).

Table 5-2: Relationship of serial postoperative values of albumin, CRP and WCC with complications grouped using the Clavien-Dindo classification (CD) for the development data set 2005-2010

	No complication (CD 0, n=84)	Minor complication (CD 1-2, n=104)	Major complication (CD 3-5, n=70)	*p value*
Preoperative Albumin	39.0 (22.0-45.0)	38.0 (20.0-44.0)	39.0 (15.0-46.0)	0.561
Albumin day 1	24.0 (16.0-32.0)	24.0 (11.0-32.0)	24.0 (10.0-37.0)	0.673
Albumin day 2	24.0 (16.0-33.0)	23.0 (14.0-32.0)	22.0 (10.0-31.0)	0.086
Albumin day 3	24.0 (16.0-32.0)	23.0 (13.0-30.0)	21.0 (9.0-32.0)	**0.008**
Albumin day 4	24.0 (15.0-34.0)	23.0 (13.0-31.0)	21.0 (9.0-30.0)	**0.002**
Albumin day 5	24.0 (17.0-37.0)	22.0 (13.0-30.0)	21.0 (12.0-30.0)	**<0.0001**
Albumin day 6	25.0 (16.0-37.0)	22.0 (15.0-31.0)	20.0 (12.0-33.0)	**<0.0001**
Albumin day 7	26.0 (15.0-39.0)	23.0 (16.0-33.0)	20.0 (13.0-31.0)	**<0.0001**
Preoperative CRP	3.0 (1.0-95.0)	4.0 (1.0-199.0)	2.0 (1.0-34.0)	0.207
CRP day 1	87.0 (24.9-233.0)	81.0 (1.0-282.0)	80.0 (7.0-268.0)	0.934
CRP day 2	224.0 (36.5-381.0)	219.0 (46.0-416.0)	224.0 (50.0-489.0)	0.604
CRP day 3	224.0 (5.0-500.0)	216.5 (49.0-406.0)	233.0 (65.0-500.0)	0.352
CRP day 4	184.0 (14.1-482.0)	187.5 (17.0-423.0)	237.0 (53.0-500.0)	0.062
CRP day 5	155.0 (6.6-388.0)	163.0 (11.0-476.0)	200.5 (18.0-500.0)	**0.005**
CRP day 6	136.5 (25.0-300.0)	144.0 (10.0-367.0)	188.0 (31.0-500.0)	0.064
CRP day 7	115.0 (21.0-377.0)	131.0 (8.0-447.0)	174.0 (16.0-500.0)	**0.035**
Preoperative WCC	7.5 (4.1-15.2)	7.3 (2.8-18.2)	6.6 (3.2-11.6)	0.179
WCC day 1	10.4 (2.7-19.5)	10.3 (3.7-49.5)	10.6 (4.3-34.5)	0.612
WCC day 2	11.2 (4.4-29.7)	11.2 (3.6-25.3)	11.6 (3.5-43.0)	0.724
WCC day 3	9.4 (4.7-29.4)	10.0 (3.3-24.2)	10.6 (4.3-34.5)	0.209
WCC day 4	7.8 (4.1-13.6)	8.2 (3.1-20.2)	9.4 (3.1-34.3)	0.071
WCC day 5	7.4 (3.1-19.7)	8.5 (3.2-32.1)	9.5 (3.6-29.3)	**0.002**
WCC day 6	8.2 (3.9-22.0)	9.4 (3.2-29.0)	10.3 (4.2-28.4)	**0.002**
WCC day 7	9.1 (4.5-25.0)	9.4 (3.7-30.8)	11.0 (4.9-41.0)	**0.006**
Preoperative NUn score	6.03 (5.05-7.47)	5.97 (4.94-8.79)	6.26 (4.82-8.19)	0.666
NUn score day 1	9.44 (7.68-11.76)	9.77 (7.54-13.84)	9.74 (7.36-14.32)	0.558
NUn score day 2	10.27 (7.88-14.18)	10.54 (8.12-12.92)	10.82 (8.11-16.92)	0.204
NUn score day 3	10.36 (8.37-14.24)	10.55 (8.11-13.42)	11.02 (8.46-15.18)	**0.016**
NUn score day 4	9.82 (7.80-11.76)	10.23 (7.59-13.86)	10.63 (8.19-14.20)	**<0.0001**
NUn score day 5	9.38 (6.33-11.23)	10.16 (7.92-14.77)	10.33 (8.26-15.04)	**<0.0001**
NUn score day 6	9.21 (6.39-12.51)	9.95 (7.17-13.44)	10.90 (7.74-14.97)	**<0.0001**
NUn score day 7	9.30 (5.88-14.06)	9.88 (7.18-15.50)	11.02 (7.93-16.42)	**<0.0001**

*p value*s were calculated using Kruskal Wallis test. Complications were defined using Clavien-Dindo classification.

Table 5-3: Relationship of serial postoperative values of albumin, CRP and WCC and anastomotic leak for the development data set 2005-2010

	No anastomotic leak (n=232)	Anastomotic leak (n=26)	*p value*
Pre-op Albumin	38.0 (20.0-46.0)	39.0 (15.0-45.0)	0.919
Albumin day 1	24.0 (10.0-37.0)	23.5 (10.0-35.0)	0.861
Albumin day 2	24.0 (11.0-33.0)	22.0 (10.0-31.0)	0.223
Albumin day 3	23.0 (9.0-32.0)	20.5 (10.0-30.0)	**0.010**
Albumin day 4	23.0 (9.0-34.0)	19.0 (13.0-30.0)	**0.001**
Albumin day 5	23.0 (12.0-37.0)	19.0 (13.0-26.0)	**<0.0001**
Albumin day 6	23.0 (13.0-37.0)	19.0 (12.0-31.0)	**<0.0001**
Albumin day 7	24.0 (13.0-39.0)	19.0 (14.0-31.0)	**<0.0001**
Preoperative CRP	3.0 (1.0-199.0)	2.0 (1.0-25.0)	0.182
CRP day 1	82.0 (1.0-282.0)	81.0 (27.0-268.0)	0.569
CRP day 2	218.0 (36.5-416.0)	272.0 (62.0-489.0)	**0.035**
CRP day 3	218.0 (5.0-500.0)	291.0 (78.0-500.0)	**0.013**
CRP day 4	187.0 (14.1-482.0)	284.0 (58.0-500.0)	**0.007**
CRP day 5	163.0 (6.6-476.0)	280.5 (18.0-500.0)	**<0.0001**
CRP day 6	143.0 (10.0-500.0)	192.5 (38.0-500.0)	**0.018**
CRP day 7	136.0 (8.0-464.0)	176.0 (37.0-500.0)	0.095
Preoperative WCC	6.9 (2.8-18.2)	7.2 (5.0-11.6)	0.468
WCC day 1	10.3 (2.7-49.5)	11.0 (6.3-34.5)	0.481
WCC day 2	11.2 (3.6-29.7)	12.0 (3.5-43.0)	0.420
WCC day 3	9.7 (3.3-29.4)	11.7 (3.8-33.6)	0.055
WCC day 4	8.1 (3.1-20.2)	11.4 (3.8-33.6)	**0.001**
WCC day 5	8.3 (3.1-32.1)	10.7 (6.1-29.3)	**0.001**
WCC day 6	8.8 (3.2-29.0)	13.5 (6.9-28.4)	**<0.0001**
WCC day 7	9.1 (3.7-30.8)	13.7 (7.9-41.0)	**<0.0001**
Preoperative NUn score	6.11 (4.82-8.79)	5.87 (5.05-6.00)	0.261
NUn score day 1	9.74 (7.36-13.84)	9.77 (7.82-14.32)	0.581
NUn score day 2	10.52 (7.88-14.18)	11.00 (8.28-16.92)	**0.011**
NUn score day 3	10.48 (8.11-14.24)	11.57 (9.47-15.18)	**<0.0001**
NUn score day 4	10.04 (7.59-13.04)	11.36 (8.86-14.20)	**<0.0001**
NUn score day 5	9.82 (6.33-14.77)	11.38 (8.64-15.04)	**<0.0001**
NUn score day 6	9.67 (6.39-13.44)	11.40 (10.33-14.97)	**<0.0001**
NUn score day 7	9.71 (5.88-15.50)	11.49 (8.85-16.42)	**<0.0001**

*p value*s were calculated using the Mann Whitney U test.

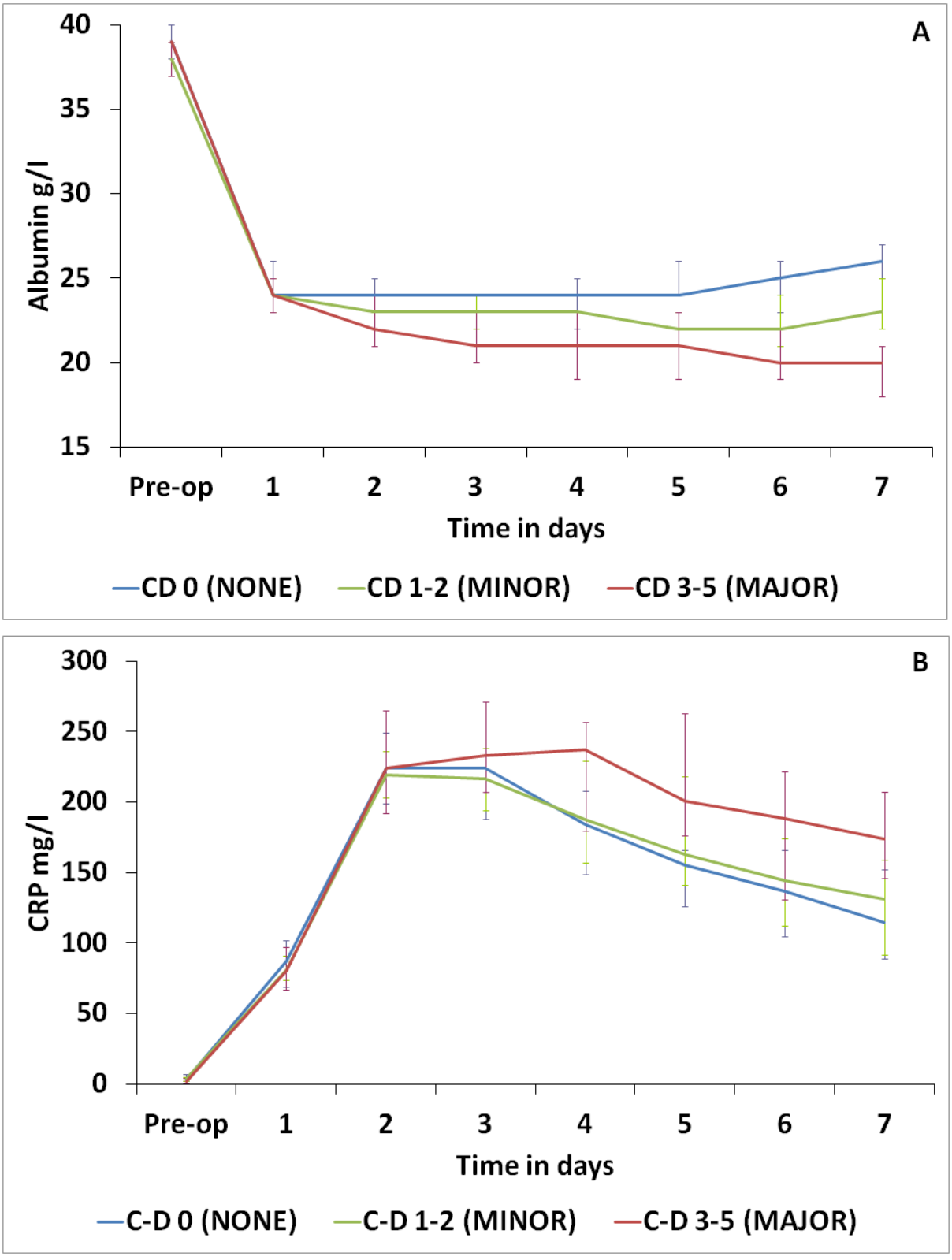
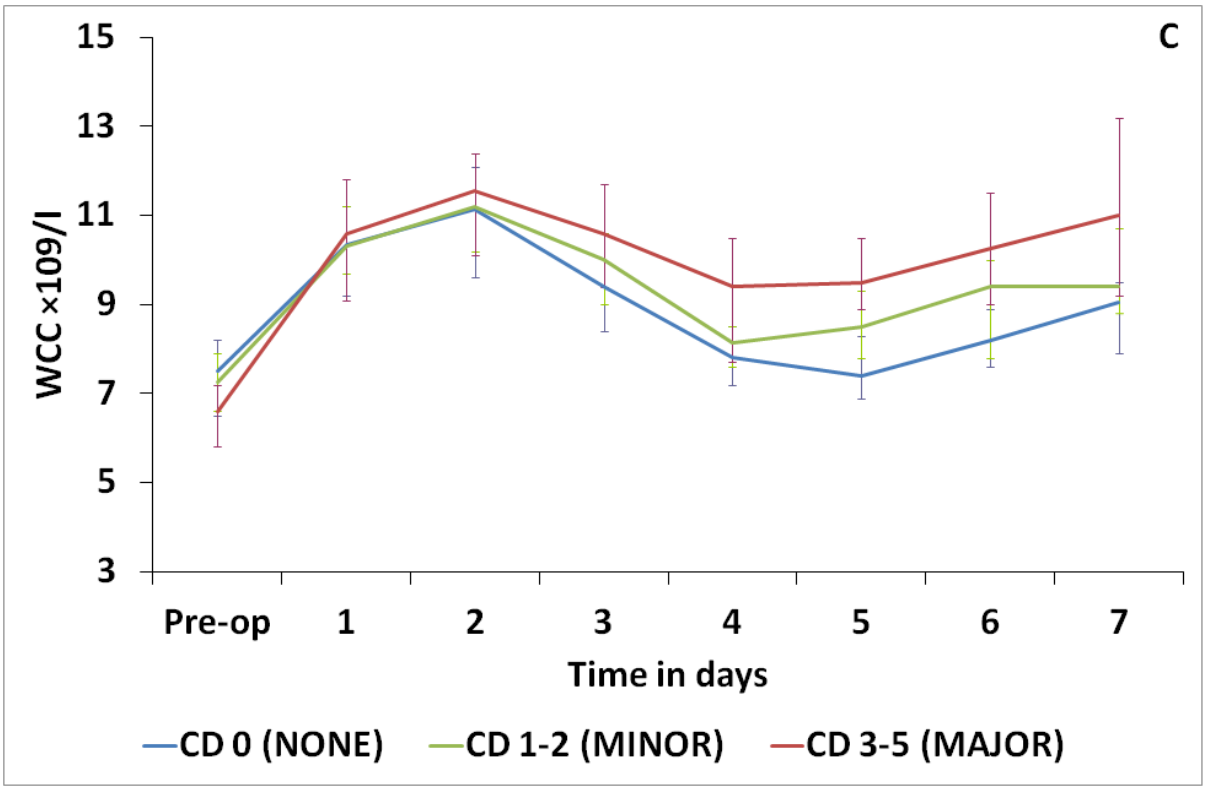


Figure 5-1: The perioperative changes in blood borne markers of systemic inflammatory response in patients grouped by complication by the Clavien-Dindo classification for the development data set 2005-2010

(A - albumin (g/l), B - CRP (mg/l), C - WCC (×109/l), and D – NUn score)



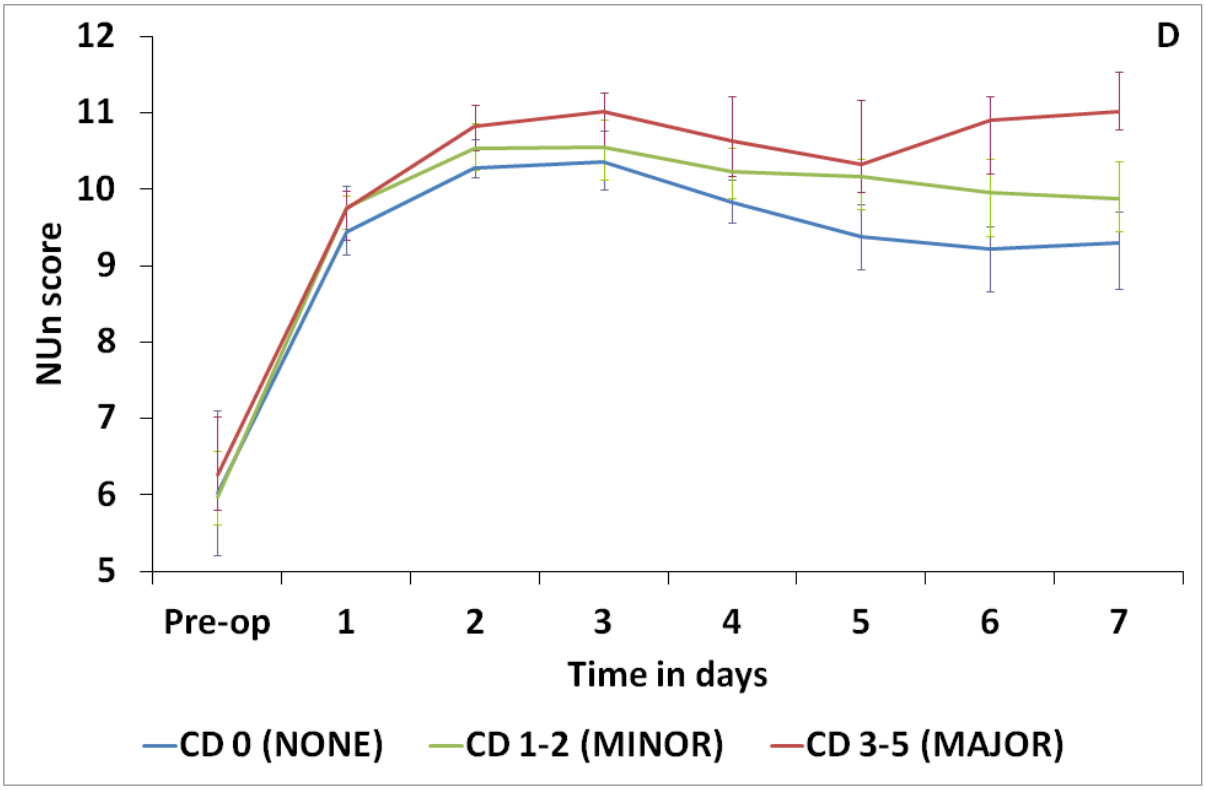
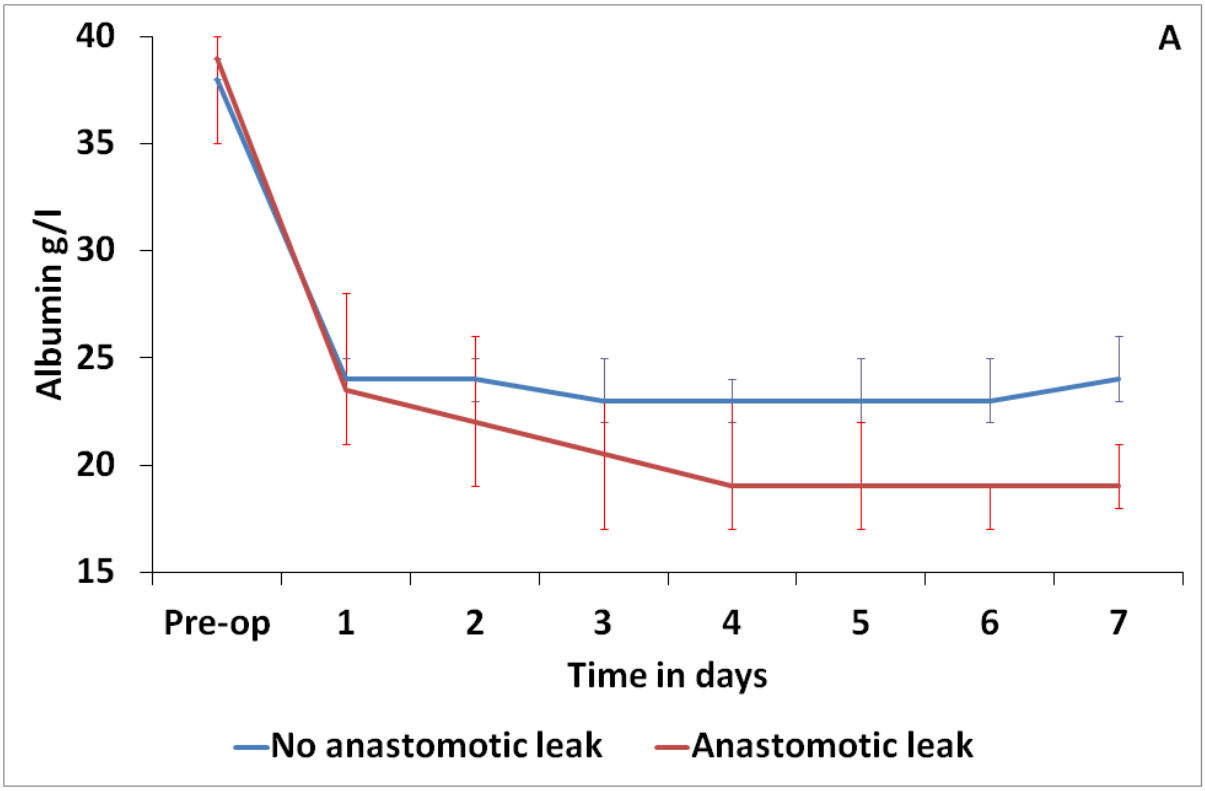


Figure 5-1: The perioperative changes in blood borne markers of systemic inflammatory response in patients grouped by complication by the Clavien-Dindo classification for the development data set 2005-2010

(A - albumin (g/l), B - CRP (mg/l), C - WCC (×109/l), and D – NUn score)



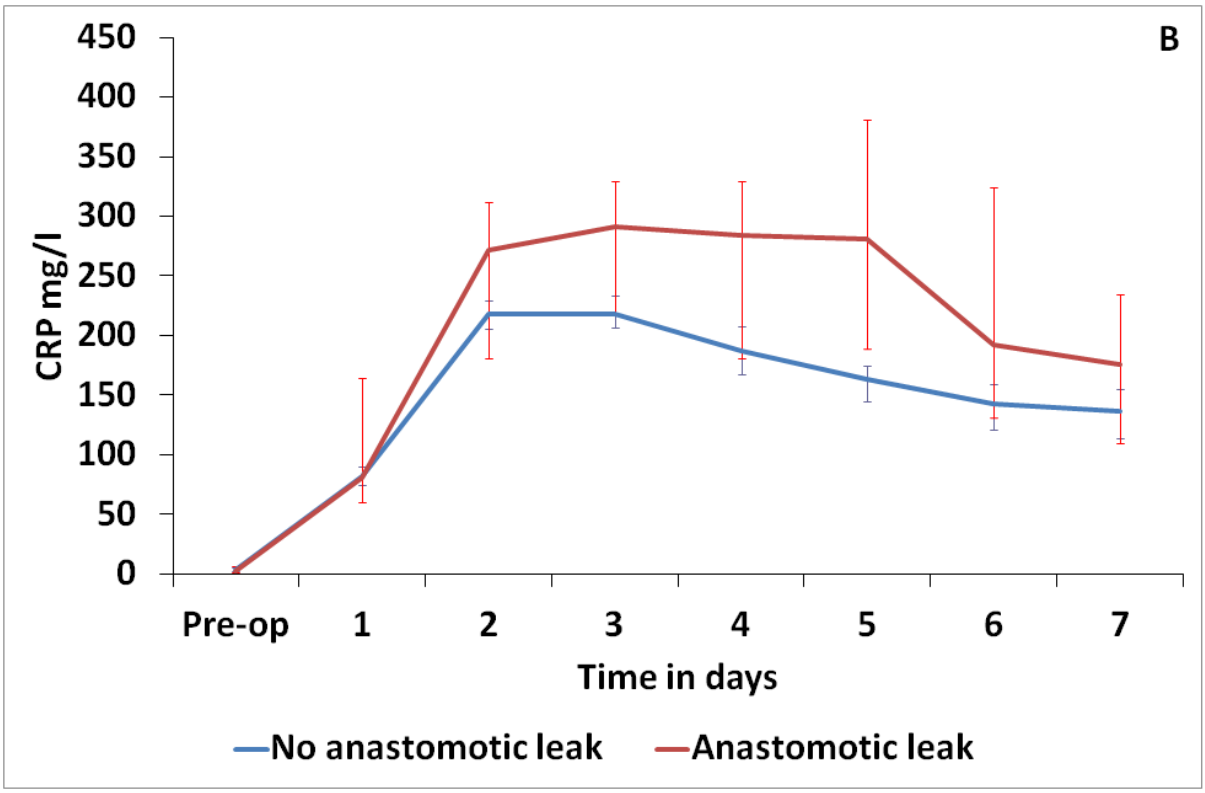
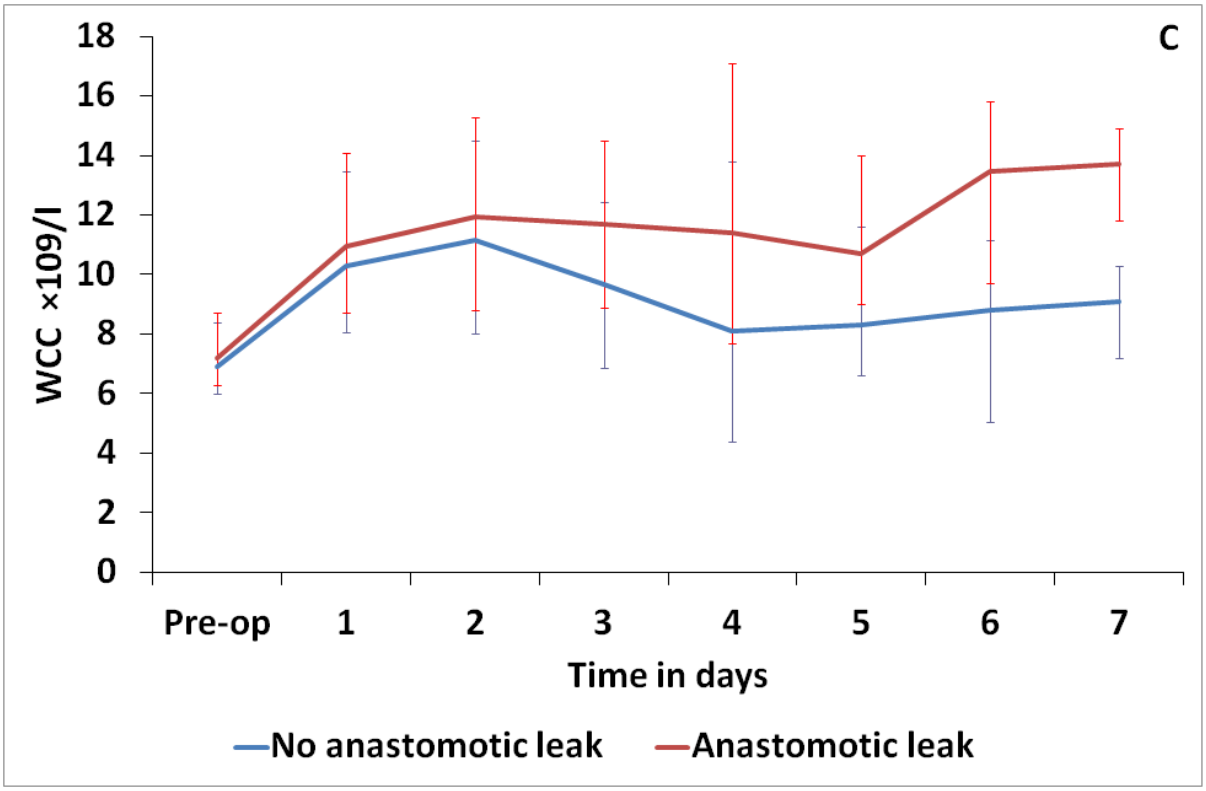


Figure 5-2: The perioperative changes in blood borne markers of systemic inflammatory response in patients with and without anastomotic leak for the development data set 2005-2010

(A - albumin (g/l), B - CRP (mg/l), C - WCC (×109/l), and D – NUn score)



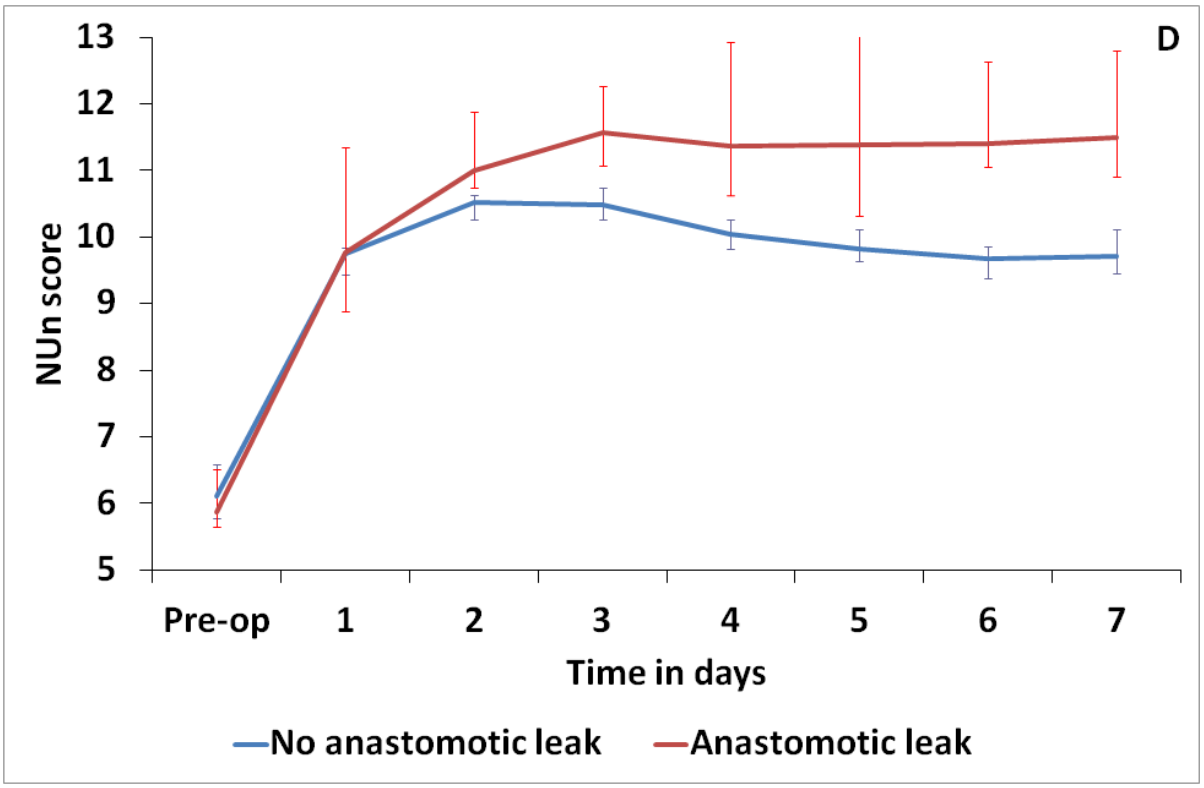


Figure 5-2: The perioperative changes in blood borne markers of systemic inflammatory response in patients with and without anastomotic leak for the development data set 2005-2010

(A - albumin (g/l), B - CRP (mg/l), C - WCC (×109/l), and D – NUn score)

Table 5-4: Univariate and multivariate binary logistic regression analysis of blood tests for anastomotic leak on postoperative day 4 for the development data set 2005-2010

	Univariate analysis			Multivariate analysis		
Variable	**Odds ratio**	**95% CI**	***p value***	**Odds ratio**	**95% CI**	***p value***
Albumin	0.831	0.745-0.928	0.001	0.840	0.739-0.955	0.008
CRP	1.008	1.003-1.013	0.002	1.005	1.000-1.010	0.062
WCC	1.253	1.130-1.389	<0.0001	1.205	1.063-1.365	0.003
NUn score	2.708	1.754-4.180	<0.0001			

NUn score =11.3894 + (0.005×CRP) + (WCC×0.186) - (0.174×albumin)

Figure 5-3: NUn score calculated using the log likelihood ratio of blood borne variables of the systemic inflammatory response (albumin, CRP and WCC from POD 4) with respect to anastomotic leak for the development data set

Table 5-5: Comparison of different threshold values of albumin, CRP, WCC and NUn score for major complications or anastomotic leak for the development data set 2005-2010

Variable	Threshold	Day	Major complications			Threshold	Anastomotic Leak		
			Sensitivity (%)	Specificity (%)	AUC (95%CI)		Sensitivity (%)	Specificity (%)	AUC (95%CI)
Albumin	>23.5 g/l	5	71	66	0.764 (0.675-0.853)	>22.5 g/l	76	56	0.742 (0.637-0.846)
CRP	>137mg/l	5	78	40	0.682 (0.577-0.787)	>188.5 mg/l	78	63	0.751 (0.611-0.891)
WCC	>8.1 $\times 10^9$/l	5	72	59	0.676 (0.580-0.772)	>8.95$\times 10^9$/l	78	58	0.715 (0.596-0.833)
NUn Score	>10	5	64	76	0.772 (0.678-0.865)	>10	88	55	0.796 (0.680-0.911)
NUn Score	>10	4	69	59	0.723 (0.627-0.818)	>10	95	49	0.801 (0.692-0.909)
CRP	>180 mg/l	4	65	47	0.613 (0.507-0.718)	>180 mg/l	75	47	0.685 (0.531-0.839)

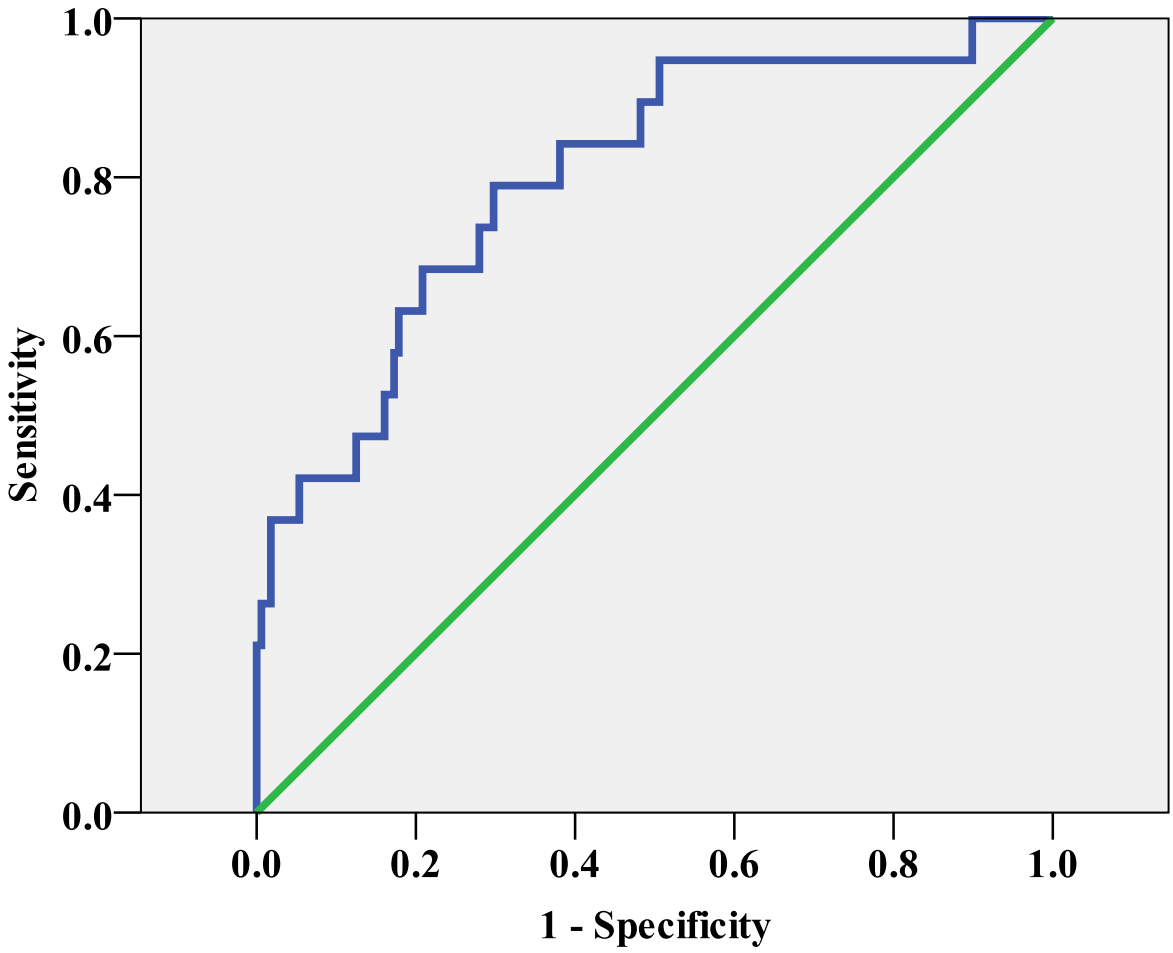


Figure 5-4: Receiver operating curve (ROC) for NUn score for on POD 4 with regard to anastomotic leak for the development data set 2005-2010 with respective area under the curve (AUC) values of 0.801 (95% CI: 0.692-0.909, p<0.0001)

5.3.2. Validation data set

5.3.2.1. Patient profile and complications

A total of 42 patients (33 men and 9 women) underwent oesophageal resection for presumed cancer or high-grade dysplasia. The median age was 69 (range 23-82) years at the time of resection. 23 (55%) patients had neo-adjuvant chemotherapy**.** A minimally invasive approach was used in 28 cases (67%). Major complications (CD III-V) were observed in 10 patients (24%). There was 1 (2.4%) in-patient death. 4 (9.5%) patients had an AL diagnosed at median POD 6 (range: 2-12). Detailed patient demographics are presented in table 5-6.

5.3.2.2. Assessment of the NUn score

Using a cut off of 10, the NUn score was assessed for its relationship to complications (major complication or death (CD III-V) and AL) using a ROC curve. The NUn score on POD 4 was predictive of an anastomotic leak (NUn score >10: sensitivity 100%, specificity 57%, diagnostic accuracy 0.879 (95% CI 0.763-0.994, *p*=0.014)) and a major complication or death (NUn >10: sensitivity 89%, specificity 63%, diagnostic

accuracy 0.856 (95% CI 0.709-1, *p*=0.001)). The NUn score was compared to individual markers of the systemic inflammatory response (albumin, CRP, WCC) and showed greater diagnostic accuracy, sensitivity, specificity and statistical significance in predicting major complication or death and AL at POD 4 (Table 5-7 and 5-8). The validation data set included complete data for 39/42 patients (93%) at POD 4. Of the three patients with missing data at POD 4, two had no complications and they had a NUn score <10 on POD 3 and 5. One patient had a major complication and a NUn score >10 on POD 3 and 5.

Table 5-6: Clinicopathological characteristics of oesophagogastric resection patients grouped by complications using the Clavien-Dindo classification in prospective validation set 2011

		No complication (CD 0, n=20)	Minor complication (CD 1-2, n=12)	Major complication (CD 3-5, n=10)
Operation age (range)		66.80 (23-82)	72.28 (56-82)	67.40 (33-79)
Sex ratio (M:F)		16:4	7 :5	10:0
ASA	**1**	2	0	0
	2	14	7	5
	3	4	5	5
Site	**Middle 1/3**	1	0	0
	Lower 1/3	10	6	3
	OGJ-S1	0	1	3
	OGJ-S2	5	3	2
	OGJ-S3	4	2	3
pT	**T0**	0	1	0
	T1	5	2	1
	T2	3	3	2
	T3	7	5	5
	T4	2	1	2
	T IS/HGD	3	0	0
pN	**N0**	14	8	4
	N1	2	1	2
	N2	0	2	2
	N3	4	1	2
pM	**M0**	20	12	10
Neoadjuvant chemotherapy		9	7	7
Surgical approach	**IVL**	2	3	2
	MIO	13	7	8
	Mckeown	1	0	0
	Transhiatal	4	2	0

Table 5-7: Relationship of serial postoperative values of WCC, albumin and CRP and NUn score with complications grouped using the Clavien-Dindo classification (CD) in prospective validation set 2011

	No complication (CD 0, n=20)	Minor complication (CD 1-2, n=12)	Major complication (CD 3-5, n=10)	*p* value
Pre-operative Albumin	38.0 (36.0-44.0)	41.0 (29.0-42.0)	37.0 (30.0-44.0)	0.789
Albumin day 1	28.0 (21.0-34.0)	29.0 (17.0-32.0)	27.0 (11.0-29.0)	0.194
Albumin day 2	26.0 (20.0-33.0)	26.5 (18.0-29.0)	23.0 (14.0-26.0)	**0.033**
Albumin day 3	24.5 (21.0-30.0)	23.5 (19.0-26.0)	21.5 (12.0-27.0)	0.052
Albumin day 4	23.5 (19.0-33.0)	22.0 (18.0-27.0)	19.5 (14.0-23.0)	**0.001**
Albumin day 5	26.0 (20.0-37.0)	22.0 (16.0-27.0)	19.5 (13.0-23.0)	**<0.0001**
Albumin day 6	26.0 (21.0-34.0)	22.5 (18.0-28.0)	19.5 (14.0-24.0)	**<0.0001**
Albumin day 7	28.0 (22.0-33.0)	23.50 (19.0-29.0)	20.0 (14.0-29.0)	**<0.0001**
Pre-operative CRP	1.0 (1.0-4.0)	9.0 (5.0-22.0)	2.5 (2.0-3.0)	0.074
CRP day 1	67.0 (2.0-221.0)	110.0 (12.0-195.0)	168.0 (50.0-337.0)	**0.008**
CRP day 2	171.0 (46.0-374.0)	226.0 (91.0-432.0)	309.5 (141.0-428.0)	**0.019**
CRP day 3	173.0 (27.0-369.0)	275.0 (121.0-496.0)	297.5 (216.0-420.0)	**0.001**
CRP day 4	149.5 (13.0-361.0)	268.0 (56.0-443.0)	335.0 (151.0-469.0)	**0.002**
CRP day 5	93.5 (11.0-227.0)	151.5 (42.0-462.0)	317.0 (88.0-500.0)	**0.002**
CRP day 6	80.0 (13.0-176.0)	192.5 (66.0-446.0)	279.0 (51.0-500.0)	**0.001**
CRP day 7	53.0 (10.0-123.0)	225.5 (62.0-379.0)	209.0 (44.0-326.0)	**<0.0001**
Pre-operative WCC	6.2 (3.2-11.7)	8.1 (5.4-11.4)	6.8 (4.6-17.5)	0.155
WCC day 1	12.0 (8.1-19.9)	12.8 (8.8-16.7)	11.3 (7.2-26.5)	0.830
WCC day 2	11.6 (4.9-17.8)	12.7 (9.9-15.8)	12.5 (7.1-26.2)	0.609
WCC day 3	8.4 (3.9-16.0)	11.0 (8.6-14.4)	10.3 (3.5-21.9)	**0.010**
WCC day 4	7.4 (4.0-11.2)	9.6 (6.0-14.7)	10.2 (4.6-14.9)	**0.035**
WCC day 5	6.9 (4.9-8.4)	9.0 (5.9-10.9)	11.6 (4.7-24.9)	**0.001**
WCC day 6	6.7 (5.5-9.5)	10.2 (6.0-15.0)	11.5 (4.8-25.5)	**0.004**
WCC day 7	6.7 (4.2-15.6)	9.9 (4.9-26.1)	11.2 (4.6-29.9)	**0.039**
Preoperative NUn score	5.98 (4.93-6.02)	6.88 (5.71-8.54)	5.76 (5.53-6.00)	0.405
NUn score day 1	9.20 (8.53-10.98)	9.13 (8.27-11.58)	10.04 (8.28-14.35)	0.294
NUn score day 2	10.04 (8.62-12.15)	10.23 (9.13-11.11)	11.27 (9.79-15.02)	**0.002**
NUn score day 3	9.79(7.86-10.94)	10.90 (9.60-11.78)	12.33 (9.23-13.49)	**<0.0001**
NUn score day 4	9.46(7.56-10.31)	10.66 (9.08-12.09)	11.38 (9.30-13.53)	**0.001**
NUn score day 5	8.63(6.67-9.79)	10.12 (8.57-11.58)	11.45 (9.43-13.91)	**<0.0001**
NUn score day 6	8.57(6.86-9.22)	10.02 (8.28-11.70)	11.53 (9.76-16.08)	**<0.0001**
NUn score day 7	8.36(6.97-9.13)	10.78 (7.91-13.63)	10.66 (9.51-15.73)	**<0.0001**
NUn score day >10	3	8	8	**0.001**
4 <10	15	4	1	

Table 5-8: Relationship of serial postoperative values of albumin, CRP, WCC and NUn score with anastomotic leak in prospective cross-validation set (2011)

		No anastomotic leak (n=38)	No anastomotic leak (n=4)	*p value*
Pre-operative Albumin		38.0 (29.0-44.0)	41.0 (38.0-42.0)	0.350
Albumin day 1		28.0 (11.0-34.0)	28.0 (21.0-29.0)	0.619
Albumin day 2		25.0 (18.0-33.0)	26.0 (14.0-26.0)	0.666
Albumin day 3		24.0 (16.0-30.0)	21.5 (12.0-27.0)	0.425
Albumin day 4		23.0 (14.0-33.0)	20.0 (18.0-22.0)	0.070
Albumin day 5		24.0 (14.0-37.0)	19.5 (13.0-23.0)	0.050
Albumin day 6		24.0 (18.0-34.0)	19.5 (14.0-24.0)	**0.048**
Albumin day 7		24.5 (17.0-33.0)	19.5 (14.0-29.0)	0.105
Pre-operative CRP		4.0 (1.0-22.0)	2.0 (2.0-2.0)	0.510
CRP_day_1		102.0 (2.0-274.0)	197.0 (50.0-337.0)	0.291
CRP_day_2		212.0 (46.0-432.0)	320.0 (179.0-362.0)	0.124
CRP_day_3		235.0 (27.0-496.0)	326.5 (216.0-364.0)	0.123
CRP_day_4		193.0 (13.0-443.0)	313.5 (151.0-469.0)	0.165
CRP_day_5		134.0 (11.0-462.0)	305.5 (88.0-500.0)	0.147
CRP_day_6		107.0 (13.0-446.0)	196.0 (51.0-500.0)	0.586
CRP_day_7		88.0 (10.0-379.0)	184.0 (44.0-321.0)	0.604
Pre-operative WCC		6.7 (3.2-17.5)	7.7 (4.9-12.7)	0.598
WCC day 1		12.0 (7.2-19.9)	19.3 (11.5-26.5)	0.068
WCC day 2		12.2 (4.9-17.8)	18.9 (11.3-26.2)	0.051
WCC day 3		9.9 (3.5-21.9)	16.8 (10.2-21.1)	**0.017**
WCC day 4		8.5 (4.0-14.7)	12.6 (5.9-14.9)	0.149
WCC day 5		7.6 (4.7-13.6)	11.6 (10.6-24.9)	**0.007**
WCC day 6		8.0 (4.8-17.4)	18.6 (9.4-25.5)	**0.008**
WCC day 7		8.7 (4.2-26.1)	22.0 (8.2-29.9)	**0.032**
Preoperative NUn score		6.00 (4.93-8.54)	5.53 (5.53-5.53)	0.500
NUn score day 1		9.21 (8.27-12.19)	9.64 (9.28-14.35)	0.247
NUn score day 2		10.21 (8.62-12.82)	11.84 (10.76-15.02)	**0.014**
NUn score day 3		10.21 (7.86-13.15)	12.45 (11.12-13.49)	**0.002**
NUn score day 4		9.88 (7.56-13.53)	11.45 (11.09-12.44)	**0.010**
NUn score day 5		9.34 (6.67-13.54)	12.77 (9.97-13.91)	**0.005**
NUn score day 6		9.04 (6.86-12.80)	13.63 (9.76-16.08)	**0.037**
NUn score day 7		9.10 (6.97-13.63)	12.46 (9.56-15.73)	**0.040**
NUn score day 4	**>10**	15	4	**0.033**
	<10	20	0	

5.4. Discussion

This study describes the development and prospective validation of a simple score for risk assessment of major complications and AL after oesophageal resection using routine blood tests. The combination of albumin, CRP and WCC in an easy to use formula was statistically significantly different between those patients who experienced an AL and those who did not from POD 2. Patients in this cohort had their risk stratified for AL and major complications with good diagnostic accuracy on POD 4. AL is considered to be the most serious complication following oesophageal resection. In keeping with previous studies (158), AL was diagnosed at median POD 7 in the development data set and POD 6 in the validation data set. Of note, clinical indicators routinely used in the

assessment of the SIRS response were not useful for differentiating between patients who developed major complications or AL and those who had an uneventful post-operative course. This may in part explain why this group and others have diagnosed AL at POD 6 and 7 despite intensive and diligent clinical evaluation (158). The accurate prediction of those patients at high risk of an AL, prior to the onset of symptoms, may allow tailored investigation and early intervention to prevent the development of significant morbidity and mortality. During the development of the NUn score we therefore chose a cut off point (>10) that gave a high sensitivity (95%) at the expense of modest specificity (49%) at POD 4, some 3 days prior to the median time of AL diagnosis.

We then validated the NUn score in a cohort of patients who underwent surgery in 2011. In this cohort no patient underwent a left thoraco-abdominal approach and the majority (67%) underwent a minimally invasive procedure, reflecting the current trend in oesophagogastric surgery. Sensitivity (100%), specificity (57%) and diagnostic accuracy (87.9%) for AL were improved in this cohort. As with all scoring systems the cut off point can be adjusted to not only take account of the optimum point of sensitivity and specificity but clinical applicability and utility. The area under the ROC curve (AUC) is a direct measure of the accuracy of a test, with an AUC>0.8 being regarded as a clinically useful test. Using a NUn score cut off point of 10 we can be confident that the test will correctly classify patients at high risk or low risk of AL in over 80% of cases. The sensitivity and specificity of the NUn score for AL are comparable to predictive tests that are in routine clinical practice, including D-Dimer for pulmonary embolus (sensitivity 88-92%, specificity 34-36%, diagnostic accuracy 56-81%) (455).

The development of a predictive model such as this is not without shortcomings. Our development dataset spans a five-year period and includes patients operated on by four different surgeons using a range of approaches to the oesophagus and stomach, including minimally invasive techniques. However, validation of the NUn score in a contemporary series suggests that it is applicable to current practice. Missing data may have a significant impact on the predicted diagnostic accuracy of a test but is rarely reported. This lack of reporting makes it difficult to interpret previous series with confidence, as it is unlikely that any large series will have complete data collection. In this series one or more points of data were missing in 28% of patients (albumin, CRP, WCC, POD 1-7), meaning that the NUn score was generated on complete data for 187 patients. However, 187 patients remains the largest reported cohort to include CRP as a predictor of postoperative infective complications after oesophagogastric surgery. A limitation of this study is the relatively small size of the validation data set, which may not be sufficient for accurate prediction. The NUn score requires validation in other patient series undergoing similar resections. However, this series is representative of the patient demographics, current standard treatment regimen and complication rates reported in the UK by other centres for potentially curable oesophagogastric cancer (456).

The study has highlighted a number of points that may have direct clinical use other than the detection of occult complications. Firstly the lack of predictive value of blood borne markers of the systemic inflammatory response on the first two postoperative days, suggest that the numbers of tests could be reduced during this time with potential cost benefit. Perhaps the most important potential benefit of our score may be as an adjunct to a protocol driven postoperative treatment algorithm where those who are scored at low risk of developing AL may be more confidently progressed to enteral nutrition. This may provide significant benefit to patients who may develop a minor complication, in whom enteral nutrition is recognised to promote wound healing and reduce complication rate (457, 458).

A question remains about what to do in the face of a positive test (NUn > 10 at POD 4). We suggest focussed clinical assessment and investigation to exclude AL and major complication, especially pulmonary problems. In our centre these patients are not progressed enterally until the presence of an AL has been assessed by water-soluble contrast radiology, CT or endoscopy, in the absence of symptoms and signs of other major complications. If the outcomes of investigations are negative and if clinical concern persists the NUn score can be recalculated at POD 5 and 6.

This study builds on previous reports to highlight the efficacy of blood borne markers of the systemic inflammatory response as predictors of major complications following upper gastrointestinal surgery. Rather than using an individual marker we choose to combine albumin, CRP and WCC to develop a novel score with 95% sensitivity and high diagnostic accuracy for AL. We suggest that the NUn score as an adjunct in the postoperative management of patients after oesophageal resection may serve to improve outcomes as part of a protocol driven perioperative treatment algorithm.

6. Chapter 6: Refining Pathological evaluation of Neoadjuvant Therapy for Adenocarcinoma of the Oesophagus and Gastro-Oesophageal Junction based on Tumour and Lymph Node Responses

6.1. Introduction

Neoadjuvant therapy followed by surgery is established as the gold standard in the management of patients with locally advanced oesophagogastric cancer. In the UK neoadjuvant chemotherapy (NAC) in conjunction with transthoracic oesophagogastrectomy is the current standard of care for these patients (82). The potential benefits of neoadjuvant therapy include: downstaging of the primary tumour and lymph nodes (89, 459-461), an increase in the resectability of the tumour (148), elimination of micrometastases (462) and increased survival (3, 186, 463, 464). A recently suggested advantage of neoadjuvant therapy and early assessment of response is the potential for assessing in vivo the chemosensitivity of the tumour and so providing information to tailor multimodal therapy (465). Both NAC and surgery are associated with considerable morbidity and mortality (3) and evidence remains inconsistent on the survival benefit for patients who undergo NAC (3, 148, 464). The most recent meta-analysis to compare NAC versus surgery alone in 1724 patients suggests a 7% survival advantage at 2 years for patients treated with NAC for adenocarcinoma (186). Patients who have a significant pathological response to neoadjuvant therapy have consistently been shown to have improved survival when compared to patients who have not had a significant response (84, 89, 466, 467). For those patients who do not have a significant pathological response, the consequences of delay to surgery and the benefits of neoadjuvant chemotherapy is not known. Furthermore, it is unclear which patients should be considered for tailored adjuvant systemic therapy.

The pathological response to chemotherapy is most widely assessed in the UK using Tumour Regression Grading (82) (TRG) as described by *Mandard et al (83)* although this has not gained universal acceptance. This system is based on the degree of fibrosis and the amount of residual cancer at the primary tumour (83). Other proposed pathological systems for measuring neoadjuvant treatment response include complete pathological response (84), size of residual tumour (85), number of residual tumour cells (86, 87), response classification system (88), size based pathological response (85) and downstaging of cT and cN stage (89). These grading systems have predominately been developed following chemoradiotherapy with few studies assessing their utility following chemotherapy in patients with oesophageal adenocarcinoma (90-93). A number of clinically important questions could be addressed by a robust and universally accepted measure of response to neoadjuvant treatment including: the ability to accurately predict an individual patient's tumour response (via molecular biomarkers or imaging) to preoperative therapy leading to non-responders proceeding directly to surgery or being considered for alternative neoadjuvant regimes; assessment of new neoadjuvant regimes, and identification of patients who are likely to benefit from adjuvant therapy.

We have therefore assessed pathological response to neoadjuvant chemotherapy by assessing the tumour response as well as the response in the lymph nodes in a large contemporary cohort of patients with oesophagogastric adenocarcinoma managed with neoadjuvant platinum based triplet chemotherapy, and describe their associations with short- and long-term outcomes. In addition we suggest a regression grading system combining both local tumour and nodal response to NAC.

6.2. Methods

6.2.1. Patients

For this retrospective study, a prospectively collected database of consecutive patients undergoing oesophagogastric resection treated at University Hospital Southampton NHS Foundation Trust (UHSFT) between January 2005 and December 2011 was reviewed. All patients were discussed at a specialist multidisciplinary team meeting (MDT). Standard staging investigations included endoscopic ultrasonography, high-resolution computed tomography, integrated fluorodeoxyglucose positron emission tomography/computed tomography (PET-CT) and staging laparoscopy, where indicated. Patients considered suitable for potential surgical resection with tumours staged as T2 N0 M0 or above were considered for neoadjuvant chemotherapy.

Neoadjuvant chemotherapy consisted of three 21-day cycles of ECF (Epirubicin 50mg/m^2, Cisplatin 60mg/m^2, both intravenously on day 1 and protracted venous infusion 5-FU 200mg/m^2 per day) or ECX (Epirubicin 50mg/m^2, Cisplatin 60mg/m^2, both intravenously on day 1 and Capecitabine 625mg/m^2 orally twice daily for 21 days) or EOX (Epirubicin 50 mg/m^2 i.v. bolus and Oxaliplatin 130 mg/m^2 i.v. infusion over 2 hours on day 1, Capecitabine 625 mg/m^2 orally twice daily for 21 days).

Surgery was performed at UHSFT after initial staging or 4-6 weeks following neoadjuvant chemotherapy. A repeat CT scan was performed, prior to surgery, for those who received chemotherapy to assess their response to chemotherapy and disease operability. Types of oesophagogastrectomies included Ivor Lewis, left thoracoabdominal with or without cervical anastomosis and transhiatal oesophagogastrectomy or minimally invasive oesophagogastrectomy (MIO) either 2 stage (MIO-2) or 3 stage (MIO-3) in accordance with recommendations arising from the consensus statement from the Association of Upper Gastrointestinal Surgeons and the Association of Laparoscopic Surgeons for introduction of MIO (392).

Data recorded included demographics, tumour characteristics, resection type, estimated blood loss (calculated from suction bottles and weighed swabs) and histopathological analysis of the surgical specimen. TNM-7 was used to report tumour stage after analysis of pathology reports (393). Pathological tumour clearance ("R"-status) was determined according the Royal College of Pathologists' system.

Postoperative complications were graded according to the Clavien-Dindo (CD) classification (444). CD grades I and II represent minor complications, grades III and IV represent major complications (grade III require radiological, endoscopic or surgical intervention, grade IV indicates life-threatening complication requiring intensive care management) and grade V indicates death. An AL was defined as a leak sufficient to cause symptoms and confirmed by radiology (contrast enhanced multi-detector CT scan with on-table oral contrast or water soluble contrast studies), endoscopy or during surgical exploration. All patients were cared for by a specialist oesophagogastric team who applied a similar perioperative regime to all patients.

Patients were routinely followed-up for 5 years post surgery according to the following protocol: 2-4 weeks post-discharge, 3 monthly for 1 year, 6 monthly for 2 years and yearly thereafter. Patients were also seen on an "as required" basis if symptomatic. Recurrence of disease during follow-up was defined as the first site or sites of recurrence with radiological or pathological confirmation. For assessment of disease free survival (DFS), recurrence was defined as time from operation to development of local, nodal (regional) and distant metastasis (whichever occurred first).

6.2.2. Factors analysed

Pathological response to chemotherapy was assessed using the tumour regression grade (TRG) system developed by Mandard *et al,* (Table 1-2, Chapter 1, section 1.3.3.3) (83, 394) and by the downstaging of lymph nodes (cN versus pN) (89). TRG was scored by one pathologist initially (ACB) prior to its introduction by all pathologists as part of routine pathological reporting.

6.2.3. Statistical analysis

Descriptive data are represented as median and range unless indicated with Kruskal-Wallis, Mann Whitney U and Pearson's chi-squared test, which were used as appropriate for comparison. Kaplan-Meier, univariate and multivariate cox logistic regression modelling were used to assess the relationship between pathological response grading systems with DFS. All factors that showed statistical significance on univariate analysis were entered to derive the final model. DFS curves of the patients were plotted by using the Kaplan-Meier method and analysed using the Log-rank test. Stratified analyses were performed based on receipt of neoadjuvant chemotherapy, nodal stage and response to chemotherapy. A p value <0.05 was considered statistically significant for all tests. Statistical analysis was performed with SPSS® version 19 (SPSS, Chicago, Illinois, USA).

6.3. Results

6.3.1. Study patients

A total of 218 patients underwent oesophageal resection during the study interval with a mean follow up of 3 years (median follow up: 2.552 95% CI: 2.022-3.081). There was a 1.8% (n=4) inpatient mortality rate.

Patients who underwent surgery alone (n=82; 37.6%) were significantly older (p<0.0001), had worse physiological status (ASA p=0.005; Performance status p=0.001; O-POSSUM p<0.0001) and lower preoperative staged disease (cT stage p<0.0001; cN stage p<0.0001) compared to patients that underwent multimodal therapy.

136 (62.4%) patients received multimodal therapy, neoadjuvant chemotherapy and surgery, with 74.3% (n=101) of patients demonstrating some signs of pathological tumour regression (TRG 1-4) with 5.9% (n=8) having a complete pathological response. 44.1% (n=60) had downstaging of their nodal stage compared to only 15.9% (n=13) who underwent surgery alone (p<0.0001).

There were no statistically significant differences in postoperative pathological tumour stage (pT, p=0.692); pN p=0.758), postoperative complications (Clavien-Dindo maximum grade, p=0.590) or completeness of resection (p=0.772) in patients that underwent multimodal therapy versus surgery alone.

Detailed patient characteristics and clinical and pathological outcomes are summarised in table 6-1, grouped by treatment.

Table 6-1: Clinical and pathological characteristics of the 218 patients operated on for gastrooesophageal adenocarcinoma, according to treatment

		Surgery only (n=82 (37.6))	Neoadjuvant chemotherapy and surgery (n=136 (62.4))	*p*-value
Preoperative Status				
Age (Range)*		74.32 (42.08-85.41)	63.76 (32.77-81.28)	<0.0001
Sex ratio (M:F)*		68 (82.9):14 (17.1)	118 (86.8):18 (13.2)	0.439
cT stage	**1**	17 (20.7)	0 (0.0)	<0.0001
	2	30 (36.6)	16 (16.0)	
	3	34 (41.5)	114 (84.0)	
	4	1 (1.2)	6 (4.4)	
cN stage	**0**	36 (43.9)	19 (14.0)	<0.0001
	1	46 (56.1)	117 (86.0)	
cM stage	**0**	80 (97.6)	134 (98.5)	0.613
	1	1 (2.4)	2 (1.4)	
Performance status	**0**	8 (11.6)	35 (25.7)	0.001
	1	51 (73.9)	96 (70.6)	
	2	10 (14.5)	5 (3.7)	
ASA	**1**	3 (3.7)	11 (8.1)	0.005
	2	56 (68.3)	106 (78.5)	
	3	23 (28)	18 (13.3)	
O-POSSUM		18 (12-30)	16 (12-26)	<0.0001
Tumour Site	**Middle 1/3**	1 (1.2)	1 (0.7)	0.418
	Lower 1/3	32 (39)	57 (41.9)	
	OGJ-S1	19 (23.2)	23 (16.9)	
	OGJ-S2	18 (22.0)	34 (25.0)	
	OGJ-S3	12 (14.6)	20 (14.7)	
Operative outcomes				
Length of operation (mins)*		255 (120-480)	261 (120-471)	0.409
Blood loss (ml)*		300 (0-2200)	318 (0-3000)	0.429
Clavien Dindo Max	**0**	26 (31.7)	53 (39.3)	0.590
	1	5 (6.1)	8 (5.9)	
	2	35 (42.7)	40 (29.6)	
	3	6 (7.3)	17 (12.6)	
	4	6 (7.3)	17 (12.6)	
	5	4 (4.9)	0 (0)	
Anastomotic leaks		8 (9.8)	9 (6.7)	0.413
Pathological outcomes				
pT	**0**	3 (3.6)	8 (5.9)	0.692
	1	23 (28)	23 (16.9)	
	2	17 (20.7)	34 (25)	
	3	34 (41.5)	66 (48.5)	
	4	5 (6.1)	5 (3.7	
pN	**0**	40 (48.8)	73 (53.7)	0.758
	1	20 (24.4)	21 (15.4)	
	2	11 (13.4)	25 (18.4)	
	3	11 (13.4)	17 (12.5)	
pM	**0**	82 (100)	136 (100)	1.00
Tumour regression grade	**1**	-	8 (5.8)	n/a
	2	-	28 (20.6)	
	3	-	20 (14.7)	
	4	-	45 (33.1)	
	5	-	35 (25.7)	
Nodal Downstaged cN to pN		13 (15.9)	60 (44.1)	<0.0001
Positive nodes*		1 (0-21)	0 (0-24)	0.789
Nodal yield*		18 (4-49)	18 (3-53)	0.242
Resection clearance	**R0**	65 (79.3)	110 (80.9)	0.772
Vascular invasion		24 (29.3)	41 (30.1)	0.891
Lymphatic invasion		9 (11)	22 (16.2)	0.28
Perineural invasion		8 (9.8)	20 (14.7)	0.291
Maximum tumour diameter (mm)*		25 (0-90)	25 (0-155)	0.998
Morphology	**Ulcer**	48 (60)	96 (74.4)	0.029
	Polypoid	22 (27.5)	23 (17.8)	
	Fungating	2 (2.5)	3 (2.3)	
	Diffuse infiltrating	8 (10)	7 (5.4)	
Grade	**G1**	6 (7.3)	16 (11.8)	0.669
	G2	30 (36.6)	37 (27.2)	
	G3	46 (56.1)	82 (60.3)	
	G4	0 (0)	1 (0.7)	
Sites of recurrence	**Local**	3 (3.7)	8 (5.9)	0.461
	Nodal	5 (6.1)	14 (10.4)	0.281
	Distant	18 (22.0)	44 (32.6)	0.093

Values in parentheses are percentages unless indicated. *Values in parentheses are range

6.3.2. The relationship of TRG and clinicopathological characteristics

The relationship between patient and tumour characteristics and response to neoadjuvant chemotherapy, as defined by tumour regression grade, are presented in Table 6-2.

Of the 136 patients that underwent NAC 36 (26.5%) patients had a significant pathological response (TRG 1-2; responders) compared to 100 (73.5%) patients with no significant pathological response (TRG 3-5; non-responders). Responders and non-responders had similar preoperative clinical features (age, sex and physiological status) and clinical stage of disease (cT stage, p=0.396; cN stage, p=0.987; cM stage, p=0.456), yet responders had markedly reduced ypT stage (p<0.0001), maximal pathological tumour diameter (p<0.0001), and ypN stage (p<0.0001) and were more likely to have their nodal stage downstaged (p<0.0001) compared to non-responders (Table 6-2). In addition, responders had tumours that were more likely to be ulcers (p=0.003), showing less vascular (p=0.004), lymphatic (p=0.338) and perineural invasion (p=0.072) compared to non-responders.

Complete resection (R0) was achieved in 97.2% (n=35) of responders compared with 75% (n=75) of non-responders. There was no significant difference in postoperative complications as classified by the Clavien Dindo system, nodal yield, blood loss or operative time between groups.

6.3.3. The relationship of TRG and lymph node downstaging with Disease Free Survival

There was a significant difference in survival between responders compared to non-responders, shown in figure 6-1 A and B (Mean DFS; TRG1-2: 5.064 years 95% confidence interval (CI): 4.560-5.569 (Median DFS: not reached) versus TRG 3-5: 2.759 years 95% CI: 2.193-3.325; p<0.0001 (Median DFS: 1.613 95% CI: 0.834-2.39)).

There was no statistically significant difference in survival between patients graded as TRG 1 compared to TRG 2 (Mean DFS; TRG1: 5.021 95% CI: 4.069-5.973 versus TRG 2: 4.983 95% CI: 4.069-5.973; p=0.944 (Median DFS's: not reached)).

Patients with lymph node downstaging following NAC had improved DFS versus patients without downstaging, figure 6-2 (median DFS; LN downstaged: 5.316 years 95% CI: 4.504-6.127 (Median DFS: 5.544) versus LN not downstaged: 2.118 years 95% CI: 1.594-2.643 (Median DFS: 1.210; 95% CI: 1.026-1.394, p<0.0001).

Table 6-2: Clinical and pathological characteristics of the 136 patients treated with neoadjuvant chemotherapy for gastro-oesophageal adenocarcinoma, classified as responders (TRG 1-2) or non-reponders (TRG 3-5)

		Tumour regression grade (TRG) grouped		
		TRG 1-2 (n=36 (26.5))	**TRG 3-5 (n=100 (73.5))**	***p*-value**
Preoperative Status				
Age (Range)*		65.27 (26.99-76.04)	63.51 (32.77-81.28)	0.410
Sex ratio (M:F)*		32 (88.9):4 (11.1)	86 (86):14 (14)	0.662
cT stage	**1**	0 (0)	0 (0)	0.396
	2	2 (5.6)	14 (14)	
	3	33 (91.7)	81 (81)	
	4	1 (2.8)	5 (5)	
cN stage	**0**	5 (13.9)	14 (14)	0.987
	1	31 (86.1)	86 (86)	
cM stage	**0**	35 (97.1)	99 (99)	0.456
	1	1 (2.8)	1 (1)	
Performance status	**0**	12 (33.3)	23 (23)	0.225
	1	23 (63.9)	73 (73)	
	2	1 (2.8)	4 (4.0)	
ASA	**1**	2 (5.6)	9 (9.1)	0.408
	2	32 (88.9)	74 (74.7)	
	3	2 (5.6)	16 (16.2)	
O-POSSUM*		15 (12-23)	16 (12-26)	0.476
Tumour Site	**Middle 1/3**	1 (2.8)	0 (0)	0.738
	Lower 1/3	15 (41.7)	42 (42)	
	OGJ-S1	7 (19.4)	16 (16)	
	OGJ-S2	9 (25)	25 (25)	
	OGJ-S3	4 (11.1)	16 (16)	
Operative outcomes				
Length of operation (mins)*		262 (163-427)	260 (120-471)	0.513
Blood loss (ml)*		300 (0-3000)	325 (0-1700)	0.673
Clavien Dindo Max	**0**	14 (38.9)	39 (39.4)	0.531
	1	2 (5.6)	6 (6.1)	
	2	14 (38.9)	26 (26.3)	
	3	4 (11.1)	13 (13.1)	
	4	2 (5.6)	15 (15.2)	
	5	0 (0)	0 (0)	
Anastomotic leaks		1 (2.8)	8 (8.1)	0.276
Pathological outcomes				
pT	**0**	8 (22.2)	0 (0)	<0.0001
	1	11 (30.6)	12 (12)	
	2	9 (25)	25 (25)	
	3	8 (22.2)	58 (58)	
	4	0 (0)	5 (5)	
pN	**0**	34 (94.4)	39 (39)	<0.0001
	1	0 (0)	21 (21)	
	2	2 (5.6)	23 (23)	
	3	0 (0)	17 (17)	
pM	**0**	36 (100)	100 (100)	0.579
Nodal Downstaged cN to pN		30 (83.3)	30 (30)	<0.0001
Positive nodes*		0 (0-5)	1 (0-24)	<0.0001
Nodal yield*		18 (4-25)	18 (3-53)	0.984
Resection clearance	**R0**	35 (97.2)	75 (75)	0.004
Vascular invasion		4 (11.1)	37 (37)	0.004
Lymphatic invasion		4 (11.1)	18 (18)	0.338
Perineural invasion		2 (5.6)	18 (18)	0.072
Maximum tumour diameter*	**(mm)**	15 (0-110)	30 (0-155)	<0.0001
Morphology	**Ulcer**	30 (93.8)	66 (68)	0.003
	Polypoid	2 (6.3)	21 (21.6)	
	Fungating	0 (0)	3 (3.1)	
	Diffuse infiltrating	0 (0)	7 (7.2)	
Grade	**G1**	8 (22.2)	8 (8)	0.104
	G2	9 (25)	28 (28)	
	G3	19 (52.8)	63 (63)	
	G4	0 (0)	1 (1)	
Sites of recurrence	**Local**	0 (0)	8 (8.1)	0.080
	Nodal	1 (2.8)	13 (13.1)	0.082
	Distant	2 (5.6)	42 (42.4)	<0.0001

Values in parentheses are percentages unless indicated. *Values in parentheses are range

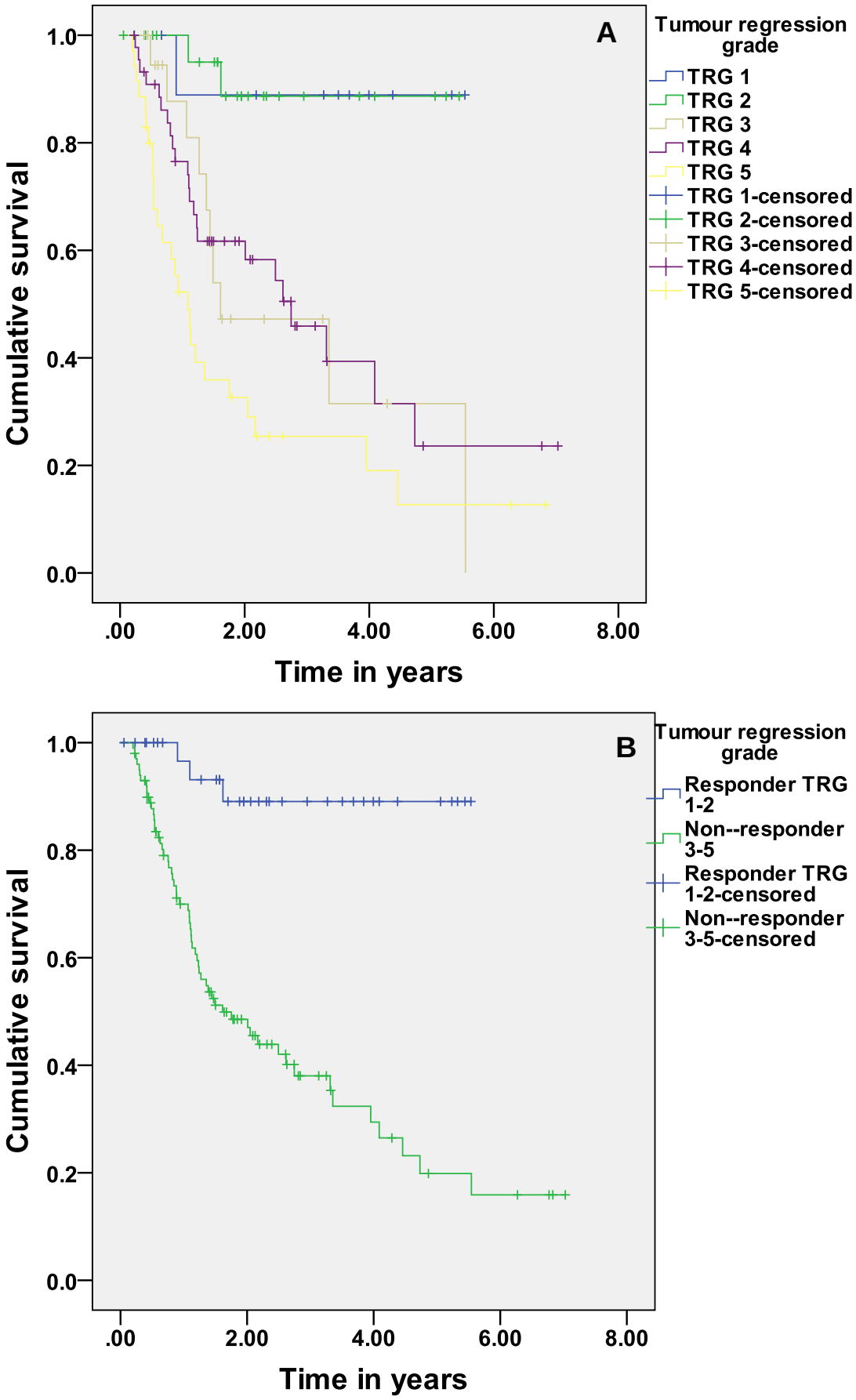


Figure 6-1: Kaplan-Meier curves of patients who received neoadjuvant chemotherapy (n=136) grouped by tumour regression grade (TRG). A – TRG 1-5 (p<0.0001). B TRG 1-2 versus TRG 3-5 (p<0.0001)

6.3.4. Univariate and multivariate analysis for predicting Disease Free Survival following neoadjuvant chemotherapy

Univariate and multivariate analysis confirmed known predictors of DFS in OAC that are detailed in table 6-3. Factors that retained significance for the prediction of worse DFS on multivariate analysis were: vascular invasion (HR: 1.929 95% CI: 1.034-3.6, *p*=0.039), perineural invasion (HR: 2.766 95% CI: 1.444-5.3, *p*=0.002), no significant response to NAC (HR: 6.315 95% CI: 1.261-31.616, *p*=0.025) and the absence of lymph node downstaging (HR: 6.161 95% CI: 1.683-22.554, *p*=0.006).

Table 6-3: Univariate and multivariate Cox regression analyses of patient and tumour factors with disease free survival for patients undergoing neoadjuvant chemotherapy (n=136)

		Univariate			Multivariate		
		HR	95%CI	*p*-value	HR	95% CI	*p*-value
Patient factors							
Age		0.972	(0.944-1.00)	0.054			
Sex	Female	1	Ref				
	Male	0.953	(0.453-2.005)	0.899			
ASA	1	1	Ref				
	2	0.696	(0.313-1.548)	0.374			
	3	0.947	(0.352-2.546)	0.914			
Performance status	0	1	Ref				
	1	1.016	(0.578-1.789)	0.955			
	2	0.950	(0.218-4.129)	0.945			
O-POSSUM							
Tumour response							
TRG	1	1	Ref				
	2	1.099	(0.099-12.148)	0.939			
	3	8.404	(1.071-65.929)	**0.043**			
	4	7.829	(1.054-58.163)	**0.044**			
	5	15.422	(2.083-114.189)	**0.007**			
TRG grouped	1-2	1	Ref		1	Ref	
	3-5	9.504	(2.973-30.380)	**<0.0001**	6.315	(1.261-31.616)	**0.025**
Lymph node response							
Lymph Nodes downstaged	Yes	1	Ref		1	Ref	
	No	5.784	(3.064-10.919)	**<0.0001**	6.161	(1.683-22.554)	**0.006**
Tumour factors							
ypT stage	0	1	Ref		1	Ref	
	1	2.085	(0.232-18.711)	0.512	0.281	(0.020-3.928)	0.345
	2	5.214	(0.687-39.549)	0.110	0.286	(0.022-3.705)	0.338
	3	9.490	(1.293-69.635)	**0.027**	0.469	(0.034-6.460)	0.571
	4	52.907	(6.008-465.873)	**<0.0001**	1.519	(0.087-26.389)	0.774
ypN stage	0	1	Ref		1	Ref	
	1	4.791	(2.434-9.431)	**<0.0001**	0.476	(0.133-1.700)	0.253
	2	4.102	(2.005-8.392)	**<0.0001**	0.254	0.070-0.927)	**0.038**
	3	7.449	(3.522-15.756)	**<0.0001**	0.476	(0.129-1.755)	0.265
ypM stage	0	1	Ref		1	Ref	
	1	3.172	(1.253-8.031)	**0.015**	2.693	(0.924-7.847)	0.069
Vascular invasion	No	1	Ref		1	Ref	
	Yes	3.444	(2.080-5.702)	**<0.0001**	1.929	(1.034-3.600)	**0.039**
Lymphatic Invasion	No	1	Ref		1	Ref	
	Yes	2.201	(1.268-3.821)	**0.005**	1.253	(0.637-2.462)	0.514
Perineural Invasion	No	1	Ref		1	Ref	
	Yes	5.073	(2.896-8.886)	**<0.0001**	2.766	(1.444-5.300)	**0.002**
Resection clearance	R0	1	Ref		1	Ref	
	R1	3.869	(2.272-6.588)	**<0.0001**	1.805	(0.940-3.468)	0.076

6.3.5. The relationship of lymph node downstaging and status with clinicopathological characteristics and Disease Free Survival

Patients with no pathological lymph node involvement were compared, grouped as those who had surgery alone versus multimodal therapy, with detailed clinical and pathological characteristics presented in table 6-4 and DFS shown in figure 6-3.

Increased pre-operative clinical stage (cT stage, $p<0.0001$; cN stage, $p<0.0001$) of disease and increased nodal downstaging (NAC 82.2% versus surgery alone 32.5%, $p<0.0001$) was observed in patients who received multimodal therapy versus surgery alone despite pathological stage being similar (pT stage, $p=0.224$; pN stage, $p=1.00$).

Patients who underwent surgery alone (pN0) had increased DFS compared to patients who underwent NAC and surgery (ypN0) (mean DFS; pN0: 6.285 years 95 %CI: 5.647-6.923 versus ypN0: 5.102 years 95% CI: 4.314-5.891 (Median DFS's: not reached), $p=0.042$).

6.3.6. Evaluation of combined local tumour response (TRG) and lymph node downstaging

83.3% of responders' additionally demonstrated downstaging of their regional lymph nodes compared to only 30% of non-responders, spread across TRG 3 to 5, figure 6-4.

The presence of lymph node downstaging in apparent non-responders was associated with significantly improved DFS (median DFS; 5.544 95% CI: 3.558-7.531 versus TRG 3-5 and LN not downstaged: 1.114 95 % CI: 0.961-1.267, $p<0.0001$), figure 6-5.

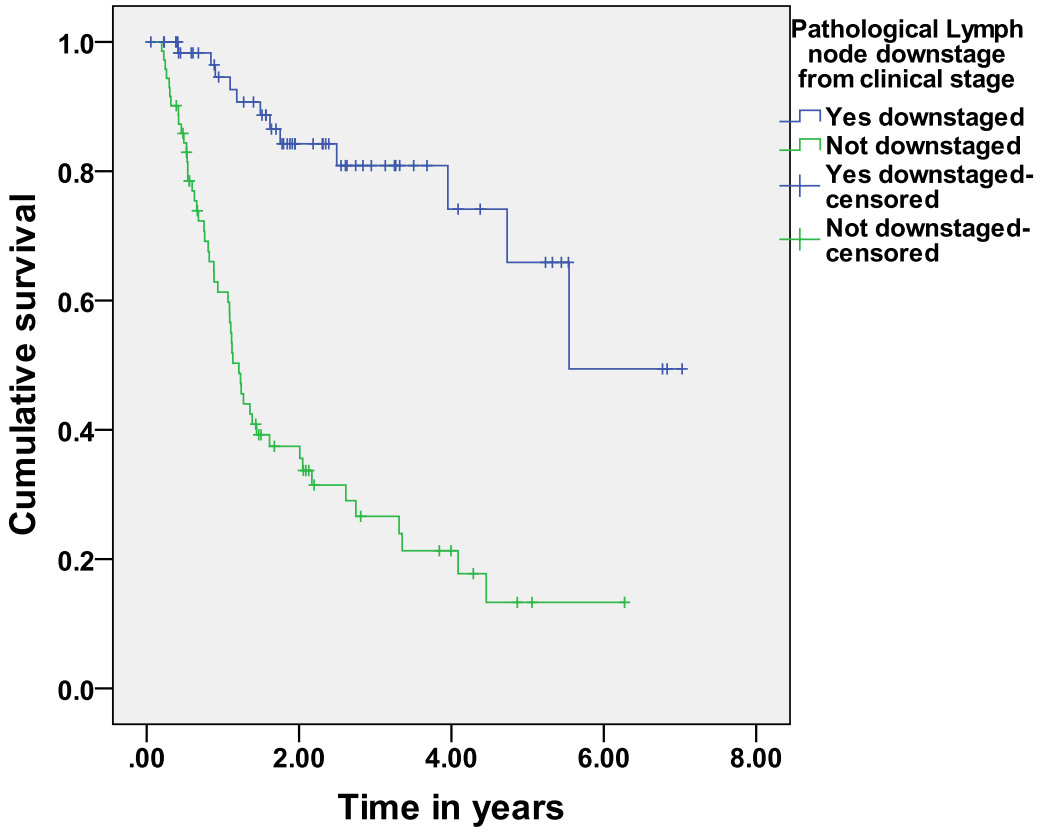


Figure 6-2: Kaplan-Meier curve of patients who received neoadjuvant chemotherapy (n=136) grouped by presence or absence of lymph node downstaging (*p*<0.0001)

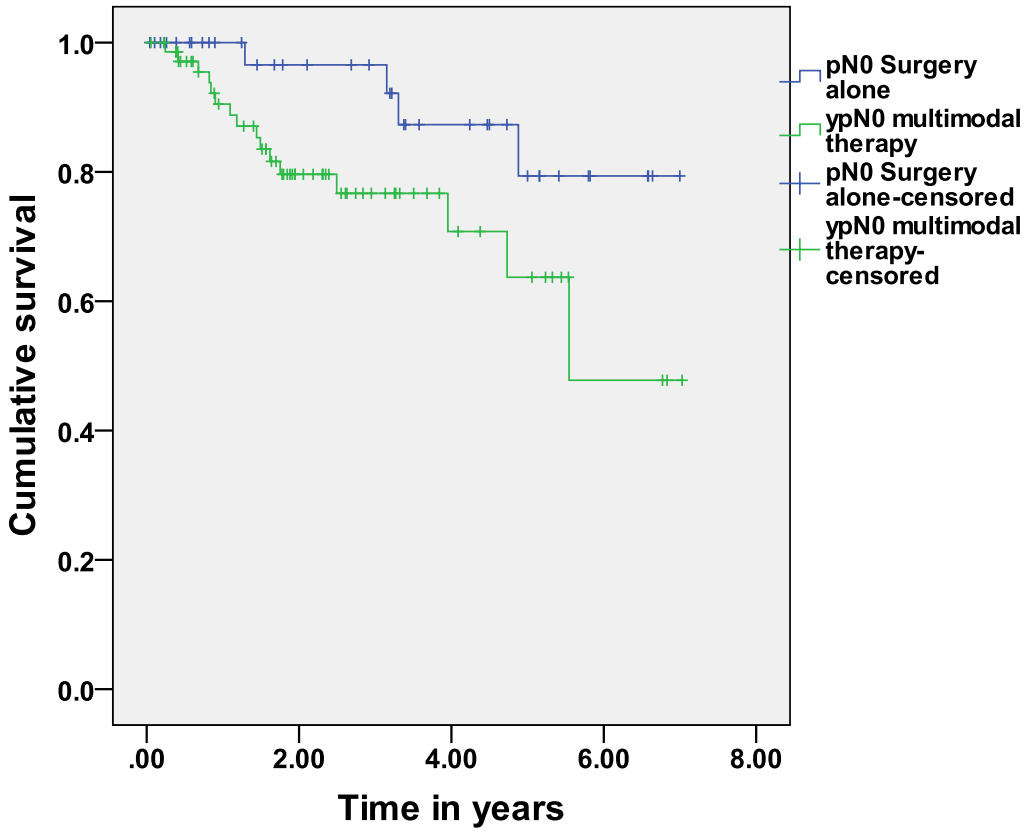


Figure 6-3: Kaplan-Meier curves of patients with no pathological lymph node metastasis (n=113) grouped by treatment (p=0.042)

Table 6-4: Clinical and pathological characteristics of the 113 patients with pathological N0 stage, according to treatment

		pN0 Surgery alone (n=40 (35.4))	pN0 Neoadjuvant chemotherapy and surgery (n=73 (64.6)	*p*-value
Preoperative Status				
Age (Range)*		73.62 (56.73-85.41)	65.59 (32.77-78.43)	<0.0001
Sex ratio (M:F)*		31 (77.5):9 (22.5)	66 (90.4):7 (9.8)	0.061
cT stage	**1**	13 (32.5)	0 (0)	<0.0001
	2	17 (42.5)	9 (12.3)	
	3	10 (25)	61 (83.6)	
	4	0 (0)	3 (4.1)	
cN stage	**0**	25 (62.5)	12 (16.4)	<0.0001
	1	15 (37.5)	61 (83.6)	
cM stage	**0**	40 (100)	71 (97.3)	0.293
	1	0 (0)	2 (2.8)	
Performance status	**0**	3 (9.4)	16 (21.9)	0.045
	1	25 (78.1)	54 (74)	
	2	4 (12.5)	3 (4.1)	
ASA	**1**	2 (5)	6 (8.2)	0.268
	2	31 (77.5)	59 (80.8)	
	3	7 (17.5)	8 (11)	
O-POSSUM*		17 (14-29)	16 (12-26)	0.015
Tumour Site	**Middle 1/3**	0 (0)	1 (1.4)	0.190
	Lower 1/3	15 (37.5)	35 (47.9)	
	OGJ-S1	11 (27.5)	12 (16.4)	
	OGJ-S2	8 (20)	16 (21.9)	
	OGJ-S3	6 (15)	9 (12.3)	
Operative outcomes				
Length of operation (mins)*		240 (120-360)	278 (120-471)	0.082
Blood loss (ml)*		200 (0-2200)	350 (0-3000)	0.167
Clavien Dindo Max	**0**	14 (35)	24 (32.9)	0.709
	1	1 (2.5)	3 (4.1)	
	2	17 (42.5)	27 (37)	
	3	4 (10)	10 (13.7)	
	4	2 (5)	9 (12.3)	
	5	2 (5)	0 (0)	
Anastomotic leaks		4 (10)	7 (9.6)	0.944
Pathological outcomes				
TRG 1-2		-	34 (46.6)	n/a
TRG 3-5		-	39 (53.4)	
pT	**0**	2 (5)	11 (15.1)	0.224
	1	22 (55)	20 (27.4)	
	2	5 (12.5)	20 (27.4)	
	3	10 (25)	24 (32.9)	
	4	0 (0)	1 (1.4)	
Nodal Downstaged cN to pN		13 (32.5)	60 (82.2)	<0.0001
Nodal yield*		16 (4-49)	18 (3-52)	0.150
Resection clearance	**R0**	35 (87.5)	69 (94.5)	0.189
Vascular invasion		7 (17.5)	10 (13.7)	0.590
Lymphatic invasion		2 (5)	6 (8.2)	0.525
Perineural invasion		2 (5)	5 (6.8)	0.698
Maximum tumour diameter (mm)*		24 (0-50)	24 (0-110)	0.324
Morphology	**Ulcer**	25 (65.8)	53 (79.1)	0.135
	Polypoid	10 (26.3)	11 (16.4)	
	Fungating	1 (2.6)	1 (1.5)	
	Diffuse infiltrating	2 (5.3)	2 (3)	
Grade	**G1**	4 (10)	13 (17.8)	0.811
	G2	17 (42.5)	20 (27.4)	
	G3	19 (47.5)	40 (54.8)	
	G4	0 (0)	0 (0)	
Site of recurrence	**Local**	0 (0)	2 (2.7)	0.293
	Nodal	1 (2.5)	4 (5.5)	0.463
	Distant	3 (7.5)	12 (16.4)	0.182

Values in parentheses are percentages unless indicated. *Values in parentheses are range

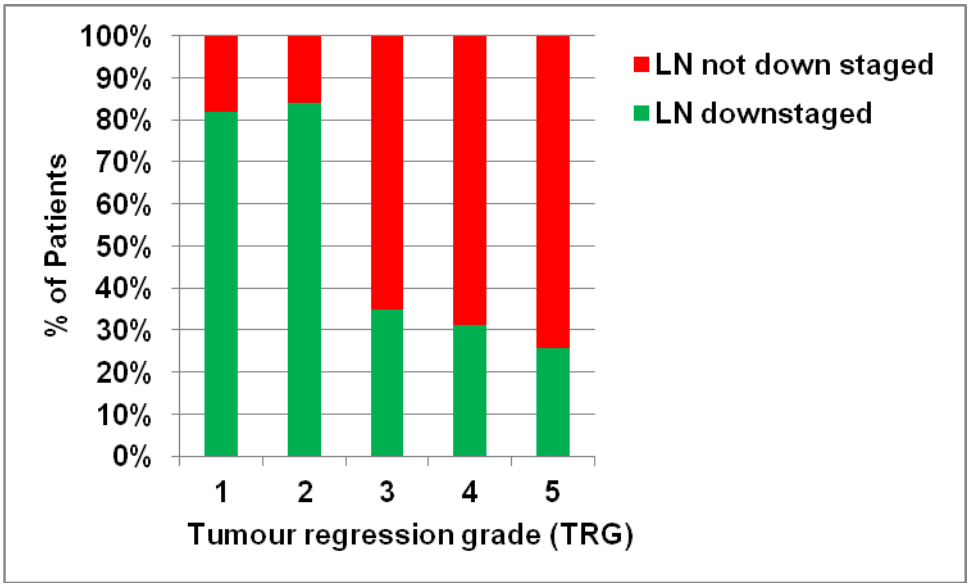


Figure 6-4: Percentage of patients who received neoadjuvant chemotherapy (n=136) having lymph node (LN) downstaging grouped by tumour regression grade (TRG)

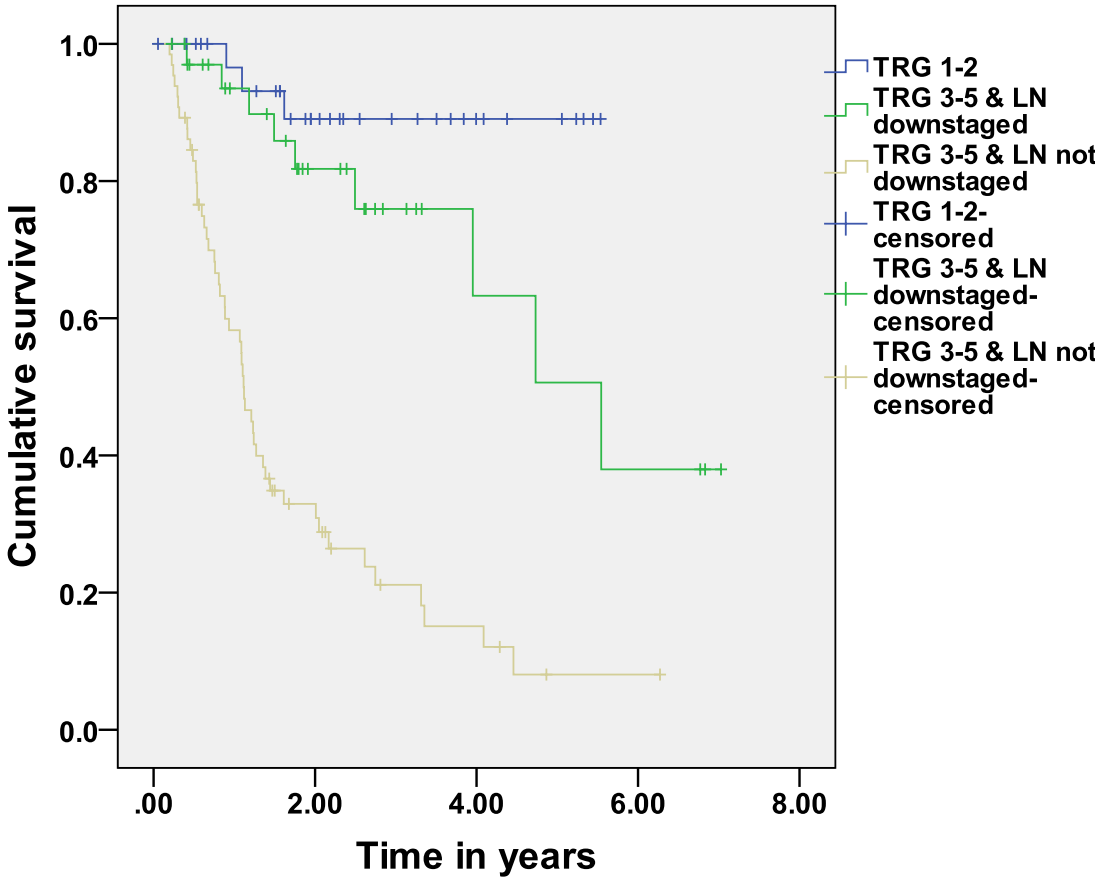


Figure 6-5: Kaplan-Meier curves of patients undergoing multimodal therapy (n=136) grouped based on a combination of tumour regression grade and lymph node downstaging (p<0.0001)

6.4. Discussion

Neoadjuvant treatment for oesophageal cancer is associated with increased survival. However, it is clear that not all patients (and their tumours) respond to neoadjuvant therapy in the same way. It is likely that improved outcomes will be observed by the tailoring of neoadjuvant therapy based on patient stratification according to tumour response.

In this study we have analysed a consecutive cohort of patients with oesophageal adenocarcinoma (OAC) undergoing treatment with curative intent to assess the primary tumour and regional lymph node response to NAC. We have described three main findings: firstly, we have confirmed that a significant pathological response as described by *Mandard et al (83)* is associated with improved DFS; Secondly we have confirmed that lymph node downstaging leads to improved DFS (89); Thirdly, and most importantly, we describe that when tumour and nodal response are combined, a group of patients who previously would have been classified as non-responders to NAC actually have significantly increased DFS.

All three findings will be discussed as there is considerable debate regarding the role of tumour regression in OAC. Conflicting opinions are evident, for what represents a significant tumour response, even within the TRG grading system (83, 85, 88, 90, 91, 468, 469). Our study confirms that TRG groups 1 and 2 only, represent significant tumour regression measured by TRG. This is in keeping with previous studies that have observed a significant increase in survival and/or metabolic response on serial PET imaging for TRG groups 1 and 2 compared to TRG groups 3 to 5 (83, 86, 88, 93, 470, 471). In our study TRG 3 tumours clearly grouped with TRG 4 and 5 and not TRG 1 and 2 tumours in terms of DFS. In addition, we found there to be no significant difference in DFS between complete pathological responders (TRG 1) versus major responders (TRG 2) consistent with other studies (83, 93). As has been previously suggested this may reflect a type II error due to insufficient sample size or represent the inherent reticence that a clinician may have to make the call of complete pathological response to neoadjuvant therapy after an operation has been performed (93). The observed increase in DFS in patients with a significant tumour response to NAC in this study may also reflect the significantly increased resectability of the primary tumour. It may also reflect the selection of tumours that are biologically more favourable as suggested by reduced vascular invasion (p=0.004), tumour morphology (p=0.003) and increased lymph node downstaging (p<0.0001).

In this study we confirmed the association between lymph node downstaging after NAC and improved DFS (89). The residual number of positive lymph nodes is consistently the most important prognostic predictor associated with survival (59, 61-64). However, the clinical significance of downstaging is controversial due to the difficulties in evaluating preoperative status. This study has the advantage of using contemporary

clinical staging based on current UK practice. The comparison of nodal stage based on pre-operative staging assessment (cN) and post-operative pathology (pN) is open to the criticism that any downstaging simply reflects over-diagnosis of lymph node metastasies on preoperative staging. To address this point we assessed the survival of patients with no positive lymph nodes in the pathological specimen, comparing NAC with surgery alone (ypN0 versus pN0). We found that patients receiving NAC with ypN0 disease had reduced DFS across all sites of recurrence compared to patients treated by surgery alone with pN0 disease. This reached statistical significance when overall DFS was assessed (p=0.042). It is therefore unlikely that our clinical staging was inadequate and suggests that the majority of patients with ypN0 disease in fact had lymph node metastasis prior to treatment. Lymph node downstaging was not included in the Response Evaluation Criteria in Solid Tumours (RECIST) 1.0 (472) classification of response to chemotherapy, which was applicable at the start of the study interval. The latest revision to the RECIST criteria (1.1, 2009) (473) may now make a more meaningful comparison of lymph node involvement by CT possible.

The increased survival observed with lymph node downstaging has important implications for the staging of OAC as neoadjuvant therapy is increasingly used. Although the final pathological stage of disease may be similar between patients treated with either multimodal therapy or surgery alone we have demonstrated that the diagnostic clinical stage and the long-term DFS of these patients are different. This would suggest revisions for the staging system for OAC to take into account the differences in outcomes for patients who have similar pathologically staged disease after multimodal therapy compared to those treated by surgery alone. This hypothesis is further supported by the results of our multivariate analysis of factors independently related to outcome in neoadjuvant chemotherapy for OAC. This showed that nodal downstaging and TRG were independent predictors of DFS but that the classical markers of disease burden, pT stage and pN stage, were only statistically significant on univariate analysis. Similar observations and suggestions have been made for patients who have undergone neoadjuvant chemoradiotherapy followed by surgery when compared to patients who underwent surgery alone (446).

There are several advantages of our study compared to other published series. This study consists of a large number of consecutive patients (n=218) of uniform histological type, with consistent clinical and pathological staging and treatment provided over a contemporary time period. The retrospective nature of this study and the use of multiple pathologists assessing TRG on an individual basis are potential limitations. However, the data was vigorously collected prospectively and the use of multiple pathologists reflects the usefulness of TRG in clinical practice and is pragmatic. A debate also remains as to what system to use to assess a local tumour response to neoadjuvant therapy (83-89). The use of TRG is not without controversy as significant tumour

regression has been reported in patients who underwent surgery alone, in up to 13.7% of cases. It has been suggested that this reflects tumour growth within abundant stroma and/or lymphocytic infiltration leading to partial tumour regression (91). While the association of lymphocytic infiltration and stromal features with survival in cancer is not new (474, 475) their association with survival in OAC is yet to be fully understood and the clinical impact is unknown (476).

Although a good pathological response of the primary tumour might be expected to represent a prognostic predictor after NAC, the low response rate observed following NAC remains problematic. In this study we observed a significant response rate of 26.5% (n=36) as assessed by TRG and 44.1% (n=60) as assessed by lymph node down staging. It seems intuitive to suggest that patients who have a partial response to NAC reflected by downstaging of lymph nodes with modest response in the primary tumour (TRG 3-5) would be the most likely to gain from adjuvant therapy. There is limited data from other disease sites to suggest only patients responding to neoadjuvant treatment benefit from further treatment (477). Adjuvant therapy has not been shown to offer a survival benefit in patients with oesophageal cancer but reports have been difficult to interpret, as they have included SCC and adjuvant radiotherapy (190, 191, 478). Some studies have shown improved survival with adjuvant therapy but concerns over toxicity and the suggestion that results were confounded due to suboptimal surgery have resulted in the lack of adoption in the UK (82, 189). However, adjuvant or perioperative chemotherapy is suggested for patients with type II and III tumours of the GOJ based on the findings of the *MAGIC* trial (82, 464), tumours that are now classified as oesophageal based on the revised TNM staging system (393). It is clear from this study that some patients with modest or no tumour response and lymph node downstaging have improved survival compared to patients with poor response in both the primary tumour and lymph nodes. This identifies a previously unrecognised group of patients who may benefit from further postoperative therapy.

One can now begin to consider an algorithm for perioperative treatment of OAC that may involve induction chemotherapy followed by early PET/CT and the curtailment of, or a change to, neoadjuvant therapy for PET non-responders. Further analysis of the primary tumour and lymph nodes after surgery would direct patients with poor tumour response to NAC but nodal downstaging to adjuvant therapy. This kind of stratified therapy will be supported by ongoing studies of biomarkers and molecular imaging. The contribution of the tumour microenvironment is also likely to offer new targets for therapy and may be the place to look to explain the different responses to therapy observed between otherwise similar tumours.

Response to NAC in the primary tumour and in the lymph nodes is associated with improved outcomes after surgery for adenocarcinomas of the oesophageal and GOJ. A previously unidentified group of patients who appear to have a poor tumoural response to

NAC (TRG 3-5) benefit substantially from NAC with nodal downstaging and increased DFS.

We propose that methods to assess the pathological response to NAC are refined so that both the response in the primary tumour and the regional lymph nodes is used to guide selection of tailored post operative treatment strategies, identify biomarkers of response to chemotherapy, provide prognostic information and assess neoadjuvant therapies.

7. Chapter 7: The Role of Systemic Inflammatory and Nutritional Blood-borne markers in predicting response to neoadjuvant chemotherapy and survival in oesophagogastric cancer

7.1. Introduction

Cancer of the oesophagus and gastro-oesophageal junction is the most rapidly increasing cancer in the west, now the sixth most common cause of cancer death, and is associated with a 5-year survival of less than 15% (2). This poor prognosis is due to late presentation with metastatic disease or patients being medically unfit. Oesophagogastrectomy is performed as the primary curative treatment for local and loco-regional disease with or without neoadjuvant therapy depending on the tumour stage. Both interventions are associated with considerable morbidity and mortality (3) and so markers that can predict poor prognosis or response to neoadjuvant therapy would be helpful in making treatment decisions.

Virchow first described the association of lymphocyte infiltration of solid tumours and it is recognised that inflammation plays a major role in tumourgenesis (316). In both squamous cell carcinoma (SCC) and adenocarcinoma (AC) of the oesophagus, chronic inflammation is thought to be the precipitating factor (19). A growing numbers of studies document the extent of the inflammatory response in solid tumours and its association with prognosis (362). Despite the evidence of tumour-host immunological interaction our understanding of this process is far from clear. It is suggested that an inappropriate host inflammatory response may result in cancer growth, invasion and metastasis via a plethora of mechanisms including subversion of host immune responses, upregulation of cytokines and inflammatory mediators, inhibition of apoptosis, promotion of angiogenesis, and damage to DNA (317). Analysis of the local tumour environment has revealed the role of the adaptive immune system in preventing tumour recurrence (479). There are now calls to combine markers of both the tumour and host inflammatory response in prognostic signatures and the classification of cancer (475).

The association of poor nutritional status and gastro-oesophageal malignancy is often assumed to be secondary to mechanical effects of the tumour (380). However the effect of sub-optimal nutritional status has also been suggested to contribute to tumour development through the subversion of tumour immunity (480). The relationship between nutritional status and systemic inflammation is likely to be complex and possibly synergistic for tumour progression. Therefore interactions to modify either have the potential to improve patient outcomes.

Preoperative biochemical markers of immunonutritional status are routinely measured in the assessment of patients during the preoperative period as well as prior to the commencement of neoadjuvant chemotherapy. A number have been suggested as blood-borne biomarkers based on different patient cohorts, diseases and outcome measures, but as yet none have been applied clinically (362-369, 371, 372, 381, 388).

The aim of this study was to perform a comprehensive analysis of routinely measured systemic inflammatory and nutritional biochemical markers (serum albumin,

white cell count (WCC), neutrophil count, lymphocyte count and platelet count) and indices of these markers (Onodera's prognostic nutritional index (PNI), neutrophil-lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR)) in a large contemporary cohort of patients undergoing oesophagogastric resections in a single institution, and describe their relationship with long-term survival and response to neoadjuvant chemotherapy, the predominate neoadjuvant therapy used in the UK.

7.2. Methods

7.2.1. Patients

For this retrospective study, a prospectively collected database of consecutive patients undergoing oesophagogastric resection with oesophageal anastomosis treated at University Hospital Southampton NHS Foundation Trust (UHSFT) between January 2005 and December 2010 was reviewed. All patients followed a contemporary treatment pathway with description of general parameters included and anlaysed included in chapter 2 (Chapter 2, 2.2.3).

7.2.2. Factors analysed

Preoperative blood samples were taken for routine laboratory analysis of full blood count (FBC) and serum albumin in the preoperative period (within 1 week of resection) and before chemotherapy (within 1 week of commencement of the 1st cycle of chemotherapy). The white cell count (WCC) (reference range 4.0-11.0 $\times 10^9$/l), platelet count (reference range 150-400 $\times 10^9$/l), neutrophil count (reference range 2.0-7.5 $\times 10^9$/l) and lymphocyte count (reference range 1.5-4.0 $\times 10^9$/l) were analyzed with an automated haematological blood analyzer (Sysmex TS-500 (Sysmex UK Ltd)). Serum concentrations of albumin (normal range: 35-48 g/l) were measured in an auto-analyzer (UniCel DxC800 (Beckmann Coulter Inc)). The coefficient of variation for these methods, over the range of measurement, was less than 2% as established by routine quality control. All patients were free from infection at the time of blood collection as determined by clinical assessment.

The NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count (reference range 0.5-5). The PLR was calculated by dividing the absolute platelet count by the absolute lymphocyte count (reference range 37.5-266.7). PNI was calculated as serum albumin (g/l) + lymphocyte count ($\times 10^9$/l) $\times$ 5 (reference range 42.5-68).

C-reactive protein (CRP) and CRP-based scores were not included in the analyses due to insufficient numbers as this test was not routine preoperatively (available in 52%) at our institution. The reference range and measurement method also varied during the study period.

7.2.3. Statistical analysis

Descriptive data are represented as median and range unless indicated with Kruskal-Wallis, Mann Whitney U and Pearson's chi-squared test used as appropriate for comparison. Kaplan-Meier, univariate and multivariate cox logistic regression modelling were used to assess the relationship between blood tests and indices of these markers (NLR, PLR, WCC, and serum albumin) with OS, DFS and response to neoadjuvant chemotherapy as outcomes. All factors that showed statistical significance on univariate analysis were entered to derive the final model using the backward stepwise likelihood ratio method. OS and DFS curves of the patients were plotted by using the Kaplan-Meier method and analysed using the Log-rank test. Stratified analyses were performed based on receipt of neoadjuvant chemotherapy and pathological response to chemotherapy. A *p* value <0.05 was considered statistically significant for all tests. Statistical analysis was performed with SPSS® version 19 (SPSS, Chicago, Illinois, USA).

7.3. Results

7.3.1. Study patients

A total of 249 patients (79% male and 21% female) underwent oesophageal resection during the study interval. There was a 30 day mortality rate of 1.3% (n=3) and these patients were excluded from further analysis. The median age at operation was 67 (range 37-85) years. 246 operations were performed for presumed cancer or high grade dysplasia with 151 (61%) patients having neo-adjuvant chemotherapy. Patient characteristics are summarised in table 6-1.

Preoperative blood tests were available for all patients except for preoperative albumin that was unavailable in 10 patients (4%). Pre-chemotherapy blood tests were available in those patients who received neoadjuvant chemotherapy at UHSFT (n=77, 51%).

The median preoperative NLR was 2.33 (range: 0.42-27.63) and 19 patients (7.7%) had a preoperative NLR >5. 40 patients (17%) had a low preoperative albumin and 88 patients (36%) had low lymphocyte count. The preoperative blood tests are detailed in table 6-3.

7.3.2. Relationship of preoperative blood tests and clinicopathological characteristics

The relationship of patient and tumour characteristics to blood borne markers of systemic inflammation and nutritional status are presented in tables 6-2 and 6-3 respectively.

A rise in neutrophil count and fall in lymphocyte count was observed with increasing pT stage but neither reached statistical significance. However, when these cell counts were combined, a significant association to increasing pT stage was found with

rising NLR (p<0.023). PNI (p<0.006) and albumin (p<0.035) fell with increasing pT stage and pN stage respectively.

An increased neutrophil count was significantly associated with vascular invasion (p=0.046) and incomplete microscopic resection (R1) (p=0.031).

7.3.3. Predictors and effects of pathological response (TRG) to chemotherapy

On univariate analysis, higher prechemotherapy serum albumin and preoperative serum albumin and PNI were predictive of a pathological response to neoadjuvant chemotherapy (TRG 1-3) (Table 6-4 and 6-5). Patients with a good response to neoadjuvant chemotherapy had a significantly higher rate of complete resection (R0: TRG 1-3 93% versus TRG 4-5 64%, p=0.001) (Table 6-4).

7.3.4. The effect of chemotherapy on blood test markers of immunonutrition

Preoperative neutrophil (p<0.0001) and platelet counts (p<0.0001) were statistically significantly lower in patients who received neoadjuvant chemotherapy compared to those who underwent surgery alone (Table 6-2).

No statistically significant differences in the immunological or nutritional markers were observed based on the patient's pathological response to neoadjuvant chemotherapy (Table 6-6).

7.3.5. Relationship of preoperative blood tests and survival

Median follow up was 3.54 years with the results of the Cox proportional hazard model for predictors of OS and DFS shown in table 6-7 and 6-8. Multivariate analysis identified increasing NLR (p<0.0001), pNstage (p<0.0001) and perineural invasion (p=0.006) to be independent prognostic factors for reduced OS. The hazard ratio (HR) for increasing NLR was 1.191 (95% confidence interval (CI): 1.092-1.298). NLR >2.5 associated with worse OS (NLR ≥2.5: mean OS 3.4 years vs. NLR <2.5: mean OS 4.6 years, p=0.002 (Figure 6-1), medians not yet reached). HR for decreased OS, for NLR ≥ 2.5 was 1.825 (95% CI 1.244-2.677, p<0.002).

Multivariate analysis identified reduced albumin (p<0.034), increasing pN stage (p<0.0001), pM stage (p=0.037), vascular invasion (p<0.0001) and microscopic incomplete resection (R1) (p=0.003) as independent prognostic factors for reduced DFS. Albumin <35 g/dl correlated with reduced DFS (albumin <35: mean DFS 3.1 years vs. albumin ≥35: mean DFS 4.3 years, p=0.042 (Figure 6-2), medians not yet reached). HR for decreased DFS for albumin <35 was for 1.776 (95% CI 1.101-2.866, p=0.018).

Table 7-1: Study cohort clinicopathological characteristics

		n=246
Operation Age*		67 (37-85)
Sex ratio (M:F)		195:51
ASA	**1**	23
	2	185
	3	36
Tumour site	**Upper 1/3**	1 (0.4)
	Middle 1/3	17 (7)
	Lower 1/3	90 (37)
	OGJ	138 (56)
Type	**SCC**	32
	AC	211
	AS	3
pT	**T0/T IS/HGD**	24 (10)
	T1	52 (21)
	T2	52 (21)
	T3	110 (45)
	T4	8 (3)
pN	**N0**	128
	N1	51
	N2	40
	N3	27
pM	**M0**	240
	M1	6
Resection clearance (R0)		194 (79)
Vascular Invasion		78 (32)
Lymphatic Invasion		38 (15)
Perineural Invasion		34 (14)
Neoadjuvant chemotherapy		151 (61)
Neoadjuvant regime	**ECX**	122 (81)
	EOX	26 (17)
	ECF	3 (2)
Tumour regression grade	**1**	13 (5)
	2	18 (7)
	3	17 (7)
	4	44 (18)
	5	35 (14)
	Not assessed	24 (10)
	Surgery only	95 (39)

Values in parentheses are percentages unless indicated. *Values in parentheses are range

ASA - American Society of Anesthesiologists physical status classification system

Table 7-2: Relationship of preoperative systemic inflammatory and nutritional markers and patient characteristics

		Neutrophil count	Lymphocyte count	Platelet count	Albumin	NLR	PLR	PNI
Study cohort		4 (0.60-22.10)	1.7 (0.5-12.40)	226 (106-594)	39 (20-46)	2.33 (0.42-27.63)	132.36 (13.47-601.25)	47.50 (24-101)
	Normal range*	236 (96)	155 (63)	224 (91.1)	196 (83.1)	225 (91.5)	233 (91.5)	189 (80.1)
	Low*	2 (0.8)	88 (35.8)	18 (7.3)	40 (16.9)	2 (0.8)	1 (0.4)	47 (19.9)
	High*	8 (3.3)	3 (1.2)	4 (1.6)	0 (0)	19 (7.7)	12 (7.7)	0 (0)
Missing data	*p*-value	**0**	0	0	10 (4)	0	0	10 (4)
Age/ years	*p*-value	**0.019**	0.746	0.532	0.367	0.077	0.754	0.556
	<75	**3.90 (.60-22.10)**	1.70 (.50-12.40)	221.00 (106.00-594.00)	39.0 (20.0-46.0)	2.27 (.42-27.63)	132.50 (13.47-601.25)	47.50 (24.00-101.00)
	≥75	**4.75 (2.00-9.00)**	1.60 (.80-3.90)	231.00 (123.00-402.00)	38.0 (21.0-44.0)	2.56 (.92-8.75)	134.96 (57.95-315.00)	46.50 (26.00-63.50)
Sex	*p*-value	0.150	0.793	**<0.0001**	0.810	0.108	0.064	0.806
	Male	4.10 (.60-22.10)	1.70 (.50-12.40)	**214.00 (106.00-594.00)**	39.0 (20.0-45.0)	2.38 (.42-27.63)	126.50 (13.47-601.25)	47.50 (24.00-101.00)
	Female	3.90 (1.40-7.70)	1.65 (.80-4.90)	**250.00 (151.00-428.00)**	39.0 (22.0-46.0)	2.15 (.50-8.63)	144.17 (57.95-400.00)	46.50 (28.50-63.50)
ASA	*p*-value	0.223	0.565	0.496	0.051	0.996	0.734	0.604
	1	3.90 (1.70-7.00)	1.70 (.50-4.20)	238.00 (136.00-394.00)	39.0 (20.0-44.0)	2.50 (.71-5.25)	145.88 (43.81-470.00)	46.50 (24.00-63.50)
	2	4.00 (.60-22.10)	1.70 (.60-4.90)	218.50 (106.00-594.00)	39.0 (21.0-46.0)	2.31 (.46-27.63)	128.26 (43.85-601.25)	47.50 (26.00-60.50)
	3	4.75 (1.80-6.90)	1.70 (.70-12.40)	223.50 (134.00-396.00)	37.0 (22.0-41.0)	2.41 (.42-9.86)	128.75 (13.47-352.86)	46.50 (27.50-101.00
Performance Status	*p*-value	0.444	0.391	0.809	0.832	0.343	0.430	0.624
0		3.85 (1.70-7.10)	1.75 (.50-3.80)	227.00 (106.00-396.00)	39.0 (23.0-45.0)	2.15 (.50-6.88)	122.89 (43.85-450.00)	48.00 (31.00-58.50)
1		4.10 (.60-22.10)	1.70 (.60-12.40)	226.00 (109.00-594.00)	39.0 (20.0-46.0)	2.43 (.42-27.63)	136.11 (13.47-601.25)	47.50 (24.00-101.00)
2		4.35 (2.10-8.00)	1.65 (1.10-3.40)	207.00 (159.00-402.00)	39.0 (22.0-41.0)	2.38 (1.40-6.15)	125.83 (75.20-242.31)	47.00 (28.50-54.00)
Smoker Preop	*p*-value	0.399	0.221	0.986	0.974	0.709	0.234	0.282
No		3.90 (1.70-22.10)	1.60 (.80-3.60)	213.00 (130.00-481.00)	39.0 (22.0-46.0)	2.45 (.50-27.63)	134.00 (63.10-601.25)	46.25 (30.50-57.00)
Yes		4.05 (1.70-8.50)	1.80 (.50-12.40)	222.50 (106.00-428.00)	39.0 (20.0-45.0)	2.38 (.42-8.75)	123.17 (13.47-470.00)	48.00 (24.00-101.00)
Treatment	*p*-value	**<0.0001**	0.661	**<0.0001**	0.706	0.078	0.092	0.326
Surgery only		**4.60 (2.00-22.10)**	1.60 (.70-12.40)	**241.50 (123.00-594.00)**	39.0 (21.0-44.0)	2.42 (.42-27.63)	142.29 (13.47-601.25)	48.00 (26.00-101.00
Neoadjuvant Rx + Surgery		**3.80 (.60-8.50)**	1.70 (.50-4.90)	**213.00 (106.00-428.00)**	38.0 (20.0-46.0)	2.24 (.46-6.67)	125.65 (43.85-470.00)	47.00 (24.00-60.50)

Values in parentheses are ranges unless indicated. *Values in parentheses are percentages

Table 7-3: Relationship of preoperative systemic inflammatory and nutritional markers and tumour characteristics

		Neutrophil count	Lymphocyte count	Platelet count	Albumin	NLR	PLR	PNI
pT	*p*-value	0.152	0.316	0.697	0.061	**0.023**	0.153	**0.006**
	T0/IS/HGD	3.60 (1.90-5.30)	2.00 (1.00-4.90)	229.50 (109.00-396.00)	39.0 (32.0-44.0)	**1.85 (.79-4.45)**	116.33 (43.81-227.00)	**49.50 (39.50-60.50)**
	T1	4.00 (1.70-22.10)	1.70 (.80-3.90)	213.00 (114.00-481.00)	39.5 (21.0-45.0)	**2.06 (.77-27.63)**	113.68 (43.85-601.25)	**49.50 (26.00-63.50)**
	T2	4.00 (1.70-9.20)	1.60 (.60-2.90)	236.50 (123.00-359.00)	38.0 (23.0-46.0)	**2.35 (.71-6.57)**	135.06 (63.46-425.00)	**46.75 (29.50-56.00)**
	T3	4.20 (0.60-9.00)	1.60 (.50-12.40)	216.00 (106.00-428.00)	38.0 (20.0-45.0)	**2.51 (.42-9.86)**	138.67 (13.47-470.00)	**46.50 (24.00-101.00)**
	T4	4.75 (2.90-6.90)	1.55 (.80-2.90)	230.50 (180.00-594.00)	38.5 (29.0-43.0)	**2.71 (1.95-6.13)**	144.62 (100.53-330.00)	**44.25 (35.50-56.50)**
pN	*p*-value	0.130	0.380	0.320	**0.035**	0.508	0.966	0.114
	N0	3.80 (1.40-22.10)	1.70 (.50-4.90)	226.00 (109.00-481.00)	**39.0 (20.0-46.0)**	2.27 (.70-27.63)	134.00 (43.81-601.25)	48.00 (24.00-63.50)
	N1	4.40 (.60-7.70)	1.70 (.70-3.40)	229.00 (106.00-428.00)	**38.0 (22.0-45.0)**	2.14 (.46-9.86)	121.76 (57.24-352.86)	47.50 (28.50-56.50)
	N2	4.70 (2.10-9.20)	1.80 (.80-3.60)	239.00 (125.00-594.00)	**39.0 (22.0-44.0)**	2.42 (.91-6.57)	126.58 (46.30-330.00)	48.00 (30.50-57.00)
	N3	4.20 (2.20-9.00)	1.50 (.60-12.40)	209.00 (130.00-364.00)	**37.0 (22.0-43.0)**	2.71 (.42-5.91)	132.86 (13.47-425.00)	45.00 (27.50-101.00)
pM	*p*-value	0.528	0.757	0.538	0.959	0.370	0.912	0.677
	M0	4.00 (.60-22.10)	1.70 (.50-12.40)	226.00 (106.00-594.00)	39.0 (20.0-46.0)	2.32 (.42-27.63)	132.22 (13.47-601.25)	47.50 (24.00-101.00)
	M1	4.60 (2.80-6.30)	1.60 (1.20-2.50)	211.50 (141.00-315.00)	39.5 (29.0-40.0)	3.15 (1.56-3.62)	149.62 (78.33-198.33)	47.00 (35.50-52.50)
Resection clearance	*p*-value	**0.031**	0.784	0.159	0.583	0.197	0.420	0.909
	R0	**4.00 (1.70-22.10)**	1.70 (.50-4.90)	227.00 (109.00-594.00)	38.0 (20.0-46.0)	2.29 (.71-27.63)	133.13 (43.81-601.25)	47.50 (24.00-63.50)
	R1	**4.60 (.60-8.00)**	1.60 (.70-12.40)	208.00 (106.00-394.00)	39.0 (22.0-45.0)	2.53 (.42-9.86)	113.85 (13.47-352.86)	47.50 (27.50-101.00)
Vascular Invasion	*p*-value	**0.046**	0.377	0.963	0.078	0.077	0.577	0.110
	Yes	**4.60 (.60-9.20)**	1.60 (.60-3.70)	222.00 (125.00-594.00)	38.0 (20.0-46.0)	2.50 (.46-9.86)	139.62 (46.30-470.00)	46.50 (24.00-58.50)
	No	**3.90 (1.40-22.10)**	1.70 (.50-12.40)	226.00 (106.00-481.00)	39.0 (21.0-45.0)	2.25 (.42-27.63)	128.67 (13.47-601.25)	48.00 (26.00-101.00)
Tumour Type	*p*-value	0.218	0.119	0.117	0.509	0.978	**0.040**	0.984
	SCC	3.75 (1.40-7.10)	1.40 (.60-4.90)	242.50 (106.00-428.00)	39.0 (22.0-46.0)	2.53 (.70-5.33)	**165.56 (64.84-425.00**	46.50 (28.50-60.50)
	AC	4.10 (.60-22.10)	1.70 (.50-3.90)	220.50 (109.00-594.00)	38.0 (20.0-45.0)	2.37 (.46-27.63)	**127.05 (43.85-601.25)**	47.50 (24.00-63.50)

Values are medians with ranges in parentheses.

Table 7-4: Clinical and pathological factors in patients that received chemotherapy based on their response to chemotherapy using tumour regression grading (TRG)

		Responder (TRG 1-3)	Non-responder (TRG 4-5)	*p* value
Age*		65.59 (36.99-74.62)	63.85 (47.56-77.75)	0.686
Sex	**Male**	34 (83)	44 (83)	0.991
	Female	7 (17)	9 (17)	
Performance status	**0**	16 (39)	15 (28)	0.156
	1	24 (59)	33 (62)	
	2	1 (2.4)	5 (9.4)	
ASA	**1**	2 (5)	5 (9.4)	0.391
	2	36 (90)	39 (73.6)	
	3	2 (5)	9 (17)	
Preop T stage	**0**	1 (2)	0 (0)	0.986
	1	0 (0)	0 (0)	
	2	4 (10)	6 (11)	
	3	24 (83)	45 (85)	
	4	2 (5)	2 (4)	
Preop N stage	**0**	5 (12)	5 (9)	0.668
	1	36 (88)	48 (90)	
Preop M stage	**0**	40 (98)	53 (100)	0.794
	1	1 (2)	0 (0)	
Differentiation	**G1 – Well**	5 (12)	4 (8)	0.478
	G2 – Moderate	13 (32)	16 (30)	
	G3 – Poor	23 (56)	33 (62)	
Type	**SCC**	2 (5)	5 (9)	0.277
	AC	39 (95)	47 (89)	
	AS	0 (0)	1 (2)	
Resection	**R0**	38 (93)	34 (64)	**0.001**
	R1	3 (7)	19 (36)	
Pre-chemotherapy	**Platelet count**	221 (113-419)	227 (129-426)	0.627
values*	**Neutrophil count**	4.30 (1.10-7.50)	4.70 (2.20-8.80)	0.350
	Lymphocyte	1.90 (0.90-2.60)	1.70 (1.00-4.00)	0.607
	Albumin	39.00 (31.00-45.00)	38.00 (29.00-43.00)	**0.040**
	NLR	2.26 (0.48-7.00)	2.65 (0.88-5.18)	0.290
	PLR	127.50 (53.81-271.00)	129.23 (56.75-305.71)	0.840
	PNI	49.50 (42.50-54.50)	46.00 (36.00-60.00)	0.189
Pre-operative	**Platelet count**	193.00 (109.00-301.00)	212.00 (106.00-396.00)	0.136
values*	**Neutrophil count**	3.50 (1.70-8.50)	3.70 (0.60	0.609
	Lymphocyte	1.80 (0.90-3.80)	1.70 (0.80-3.40)	0.622
	Albumin	39.0 (27.0-45.0)	37.0 (22.0-45.0)	**0.002**
	NLR	2.00 (0.79-6.67)	2.30 (0.46-5.50)	0.471
	PLR	107.83 (43.85-239.09)	140.00 (46.30-242.31)	0.136
	PNI	48.0 (31.50-55.00)	45.50 (28.50-56.00)	**0.011**

Values in parentheses are percentages unless indicated. *Values in parentheses are range

Table 7-5: Univariate and multivariate analysis of pre-chemotherapy factors for response to chemotherapy (TRG 1-3)

		Univariate			Multivariate		
		HR	95%CI	*p* value	HR	95% CI	*p* value
Age		0.995	(0.948-1.045)	0.839			
Sex	**Female**	1	Ref				
	Male	0.953	(0.347-2.617)	0.926			
Performance status	**0**	1	Ref				
	1	1.303	(0.593-2.862)	0.510			
	2	3.810	(0.404-35.905)	0.243			
ASA	**1**	1	Ref				
	2	0.276	(0.058-1.327)	0.108			
	3	0.733	(0.101-5.330)	0.759			
Neoadjuvant Regime	**ECX**	1	Ref				
	EOX	0.661	(0.272-1.65)	0.361			
Preop T stage	**1**	1	Ref				
	2	3.554		0.999			
	3	2.651		0.999			
	4	3.231		0.999			
Preop N stage	**0**	1	Ref				
	1	1.427	(0.544-3.744)	0.471			
Differentiation	**G1 – Well**	1	Ref				
	G2 – Moderate	1.310	(0.3335-5.117)	0.698			
	G3 – Poor	1.466	(0.411-5.226)	0.556			
Tumour Type	**SCC**	1	Ref				
	AC	0.635	(0.118-3.409)	0.596			
Pre-chemotherapy values	**Platelet count**	1.003	(0.996-1.010)	0.407			
	Neutrophil count	1.317	(0.941-1.845)	0.109			
	Lymphocyte count	1.008	(0.452-2.248)	0.985			
	Albumin	0.836	(0.712-0.982)	**0.029**	0.836	(0.712-0.982)	**0.029**
	NLR	1.231	(0.834-1.816)	0.296			
	PLR	1.004	(0.995-1.013)	0.380			
	PNI	0.897	(0.797-1.010)	0.072			

Table 7-6: Difference in peripheral blood tests compared to pathological response

	Responders (TRG 1-3) n=28	**Non-responders (TRG 4-5) n=38**	
	Difference in prechemo to postchemo	**Difference in prechemo to postchemo**	***p* value**
Neutrophil count	0.60 (-0.4.10-3.40)	0.80 (-2.30-5)	0.546
Lymphocyte count	0 (-1.20-1.30)	0.20 (-0.80-1.80)	0.188
Platelet count	28.5 (-48.0-133.0)	24.5 (-82-187)	0.856
Albumin	0 (-4-15)	0 (-7-19)	0.962
NLR	0.16 (-4.71-3.06)	0.33 (-2.21-3.36)	0.990
PLR	16.20 (-105.05-134.44)	-6.24 (-61.67-145.13)	0.344
PNI	0 (-5.50-21.50)	0.50 (-11.00-21.00)	0.418

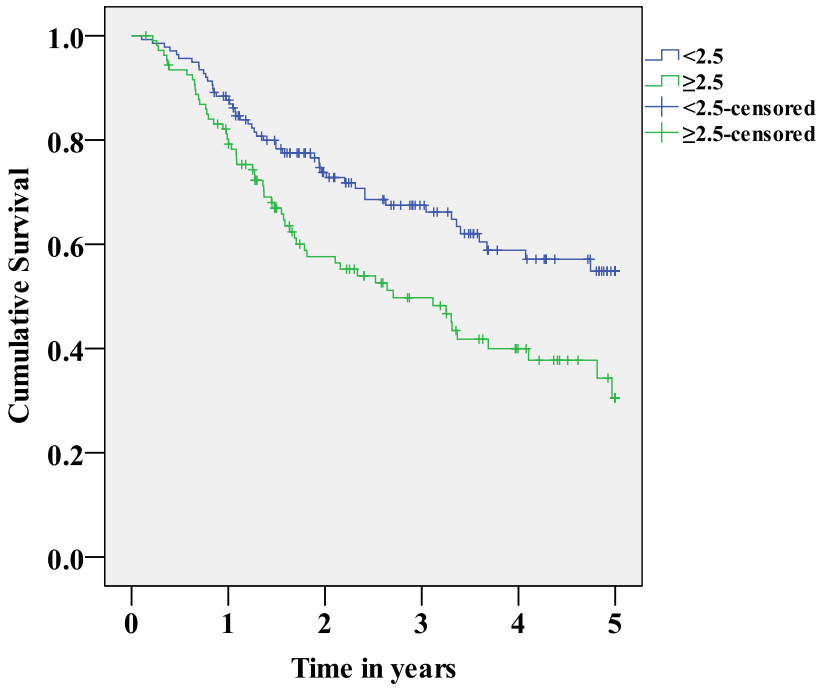
Values in parentheses are range

Table 7-7: Univariate and multivariate Cox regression analyses of patient and tumour factors with overall survival

		Univariate			Multivariate		
		HR	95%CI	*p* value	HR	95% CI	*p* value
Patient factors							
Age		0.995	(0.975-1.016)	0.666			
Sex	Female	1	Ref				
	Male	1.284	0.782-2.110)	0.323			
ASA	1	1	Ref				
	2	1.069	(0.551-2.074)	0.843			
	3	1.151	(0.526-2.516)	0.725			
Performance status	0	1	Ref				
	1	1.132	(0.704-1.820)	0.610			
	2	1.460	(0.648-3.291)	0.361			
Preoperative smoker	No	1	Ref				
	Yes	0.827	(0.493-1.385)	0.470			
Neoadjuvant Rx	No	1	Ref				
	Yes	1.387	(0.928-2.074)	0.111			
WBC		1.074	(0.982-1.175)	0.118			
Neutrophils		1.159	(1.042-1.290)	**0.007**			0.811
Lymphocytes		0.885	(0.687-1.139)	0.342			
Albumin		0.955	(0.920-0.990)	**0.012**			0.137
Platelets		1.000	(0.997-1.003)	0.837			
Patient factor scores							
NLR		1.152	(1.064-1.247)	**<0.0001**	1.191	(1.092-1.298)	**<0.0001**
PLR		1.002	(1.000-1.005)	0.056			
PNI		0.967	(0.940-0.995)	**0.020**			0.323
Tumour factors							
pT stage	0/IS/HGD	1	Ref		1	Ref	
	1	2.998	(0.664-13.527)	0.153			0.372
	2	8.045	(1.915-33.797)	**0.004**			0.506
	3	11.299	(2.754-46.349)	**0.001**			0.986
	4	31.156	(6.200-156.573)	**<0.0001**			0.154
pN stage	0	1	Ref		1	Ref	
	1	3.444	(2.057-5.768)	**<0.0001**	3.611	(2.125-6.136)	**<0.0001**
	2	4.748	(2.780-8.110)	**<0.0001**	4.010	(2.272-7.077)	**<0.0001**
	3	6.771	(3.865-11.864)	**<0.0001**	6.857	(3.775-12.456)	**<0.0001**
pM stage	0	1	Ref		1	Ref	
	1	3.160	(1.274-7.837)	**0.013**			0.185
Vascular invasion	No	1	Ref		1	Ref	
	Yes	2.463	(1.677-3.618)	**<0.0001**			0.242
Lymphatic Invasion	No	1	Ref		1	Ref	
	Yes	1.977	(1.253-3.121)	**0.003**			0.359
Perineural Invasion	No	1	Ref		1	Ref	
	Yes	3.323	(2.106-5.243)	**<0.0001**	1.980	(1.218-3.218)	**0.006**
Histology	AC	1	Ref				
	SCC	0.902	(0.513-1.585)	0.720			
Resection clearance	R0	1	Ref		1	Ref	
	R1	2.959	(1.955-4.477)	**<0.0001**			0.138

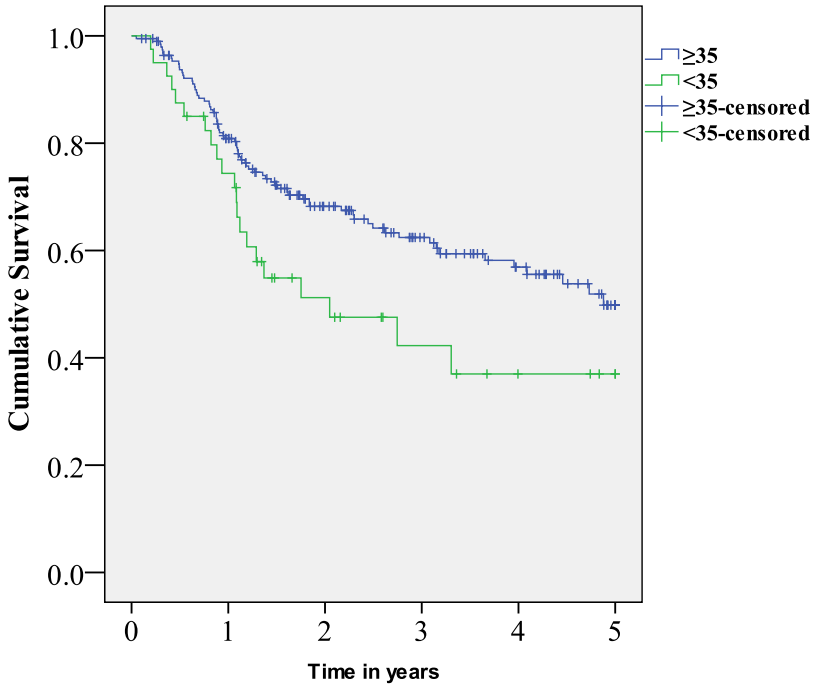
Table 7-8: Univariate and multivariate Cox regression analyses of patient and tumour factors with disease free survival

		Univariate			Multivariate		
		HR	95%CI	*p* value	HR	95% CI	*p* value
Patient factors							
Age		0.984	(0.963-1.005)	0.134			
Sex	Female	1	Ref				
	Male	1.371	(0.813-2.313)	0.236			
ASA	1	1	Ref				
	2	0.998	(0.514-1.939)	0.996			
	3	0.928	(0.412-2.092)	0.857			
Performance status	0	1	Ref				
	1	1.082	(0.670-1.748)	0.748			
	2	1.269	(0.541-2.978)	0.583			
Preoperative smoker	No	1	Ref				
	Yes	0.613	(0.362-1.040)	0.069			
Neoadjuvant Rx	No	1	Ref		1	Ref	
	Yes	1.639	(1.068-2.516)	**0.024**			0.188
WBC		1.063	(0.968-1.167)	0.200			
Neutrophils		1.096	(0.972-1.237)	0.136			
Lymphocytes		1.036	(0.845-1.271)	0.731			
Albumin		0.954	(0.919-0.990)	**0.013**	0.957	(0.919-0.997)	**0.034**
Platelets		1.000	(0.997-1.002)	0.761			
Patient factor scores							
NLR		1.070	(0.958-1.194)	0.230			
PLR		1.000	(0.997-1.003)	0.841			
PNI		0.979	(0.950-1.009)	0.165			
Tumour factors							
pT stage	0/IS/HGD	1	Ref		1	Ref	
	1	4.894	(0.620-38.638)	0.132			0.350
	2	17.716	(2.409-130.300)	**0.005**			0.142
	3	22.106	(3.054-160.032)	**0.002**			0.816
	4	43.800	(5.073-378.200)	**0.001**			0.706
pN stage	0	1	Ref		1	Ref	
	1	5.089	(2.989-8.663)	**<0.0001**	3.786	(2.162-6.630)	**<0.0001**
	2	5.863	(3.323-10.342)	**<0.0001**	3.678	(1.975-6.849)	**<0.0001**
	3	7.984	(4.294-14.843)	**<0.0001**	4.670	(2.316-9.413)	**<0.0001**
pM stage	0	1	Ref		1	Ref	
	1	3.808	(1.534-9.448)	**0.004**	2.785	(1.063-7.299)	**0.037**
Vascular invasion	No	1	Ref		1	Ref	
	Yes	3.389	(2.276-5.047)	**<0.0001**	2.173	(1.419-3.326)	**<0.0001**
Lymphatic Invasion	No	1	Ref				
	Yes	2.575	(1.652-4.013)	**<0.0001**			0.522
Perineural Invasion	No	1	Ref				
	Yes	3.009	(1.848-4.898)	**<0.0001**			0.330
Histology	AC	1	Ref				
	SCC	0.828	(0.452-1.517)	0.542			
Resection clearance	R0	1	Ref		1	Ref	
	R1	3.147	(2.061-4.803)	**<0.0001**	2.001	(1.263-3.171)	**0.003**



No. at Risk	0	1	2	3	4	5
NLR<2.5	138	118	76	53	34	16
NLR≥2.5	108	83	48	33	19	8

Figure 7-1: Overall survival according to preoperative NLR (Logrank p=0.002)



No. at Risk	0	1	2	3	4	5
Albumin<35	40	28	14	8	4	2
Albumin≥35	196	147	93	64	43	19

Figure 7-2: Disease free survival according to preoperative albumin (g/dl) (Logrank *p*=0.017)

7.4. Discussion

In this study we have analysed a consecutive cohort of patients with oesophagogastric cancer undergoing treatment with curative intent to assess blood-borne markers of the preoperative systemic inflammatory response and nutritional status. We have described three major findings; firstly, we have confirmed the association between routinely measured blood-borne markers of nutritional status and systemic inflammatory response with survival. Secondly, we showed malnutrition (as measured by PNI) to be both common preoperatively and inversely associated with the systemic inflammatory response. Finally, we found hypoalbuminemia before the start of chemotherapy corresponded to a lack of pathological response to neoadjuvant chemotherapy.

In keeping with previous studies we found pN stage to be the most significant prognostic indicator of survival (59, 61-64). We also found pM stage (67), perineural invasion (72), vascular invasion (63, 64, 73) and completeness of surgical resection (R1) (76, 77) to be independently associated with OS and DFS. These findings confirm our cohort to be truly representative of patients undergoing a contemporary radical treatment pathway for oesophagogastric cancer. It is therefore likely that our findings of significant associations between markers of systemic immunonutrition and survival are applicable to current patient cohorts.

This study demonstrates higher NLR to be an independent predictor of worse OS (HR: 1.191, 95% CI: 1.092-1.298, p<0.0001). NLR is an accessible, inexpensive measure of systemic inflammation that has been investigated as a prognostic marker in a range of solid organ tumours (362), following coronary artery bypass grafting (481) and percutaneous coronary interventions (482), and after major vascular surgery (483). NLR has been hypothesised to reflect the balance of the activation of inflammation (neutrophilia) and the cortisol-induced stress response (lymphopenia) in the acute setting (483), but may also be influenced by the relative lymphopenia of malnutrition (484). A low lymphocyte count is recognised as a predictor of poor survival in patients with advanced cancer, including gastric cancer, and is attributed to the role of lymphocytes in cell-mediated immunity causing the destruction of cancer cells (485). It is likely that the mechanisms will be more complex but NLR is thought to indirectly reflect tumour burden, invasion, and metastasis (486) through the local tumour host interaction mediated by cytokines and growth factors. Studies looking at colorectal cancer have suggested that the systemic inflammatory response is associated with changes in type, density and location of immune cells within the tumour microenvironment, providing a favourable environment for tumour invasion and metastasis (487, 488). Roxburgh *et al* noted that increased circulating white cells including neutrophils corresponded to a low peritumoral immune cell infiltrate and increased stage of disease (489). These studies suggest it would be beneficial to explore the association of the systemic and local inflammatory

response and the tumour microenvironment with a view to the development of novel therapies.

This study is not the first to assess the association of biochemical markers of nutritional status and the systemic inflammatory response with survival in cancer patients with solid tumours (361-374). Preoperative neutrophil count (363), lymphocyte count (363), platelet count (364, 365), albumin (366), CRP (362, 367, 368), NLR (362, 369, 370), PLR (362, 371), PNI (362, 372) and Glasgow prognostic score (GPS) (373, 374, 387, 490) and modified Glasgow prognostic score (mGPS) (362) have been associated with changes in survival in oesophagogastric cancer patients. However, a common feature of all these studies was the lack of uniformity in the survival outcome chosen as well as differences in the cut-off value for the proposed marker. For example, different studies reported NLR cut-off values ranging from 2 (369) to 5 (370) to be associated with long-term survival, while other studies have looked at patients with heterogenous histology (adenocarcinoma (n=244 (49.3%) and squamous cell carcinoma (n= 251, 50.7%) (387).

We choose not to dichotomise continuous data when undertaking regression analyses, firstly as multiple differing cut-off points are reported in the literature and secondly to minimise false positive results (491, 492). Our findings suggest a correlation between increasing NLR and worse OS across the continuous range of NLR. If this is proven, interventions to improve NLR may be applicable to all patients and not just those with an NLR above a pre-defined cut-off value.

Oesophagogastric cancer is often associated with preoperative malnutrition (493) and the mechanical effects of the tumour causing dysphagia may cause this. In addition, it has been proposed that increased metabolic demands and the increased production of biological mediators due to advanced tumour stage contribute to malnourishment (380). We observed 40 patients (17%) to have a low albumin preoperatively and 47 patients (20%) with a low PNI, confirming evidence of poor biochemical nutritional status as a significant problem in this patient group. There was a clear association between an increase in systemic inflammation and a fall in biochemical nutritional status with advance tumour stage in our cohort, as measured by NLR, albumin and PNI (Table 4) (380). It remains unclear as to whether the observed hypoalbuminaemia and low PNI in this cohort reflects systemic inflammation (387, 494), poor nutritional status or more likely a combination of the two. It has previously be suggested that the presence of increasing systemic inflammation and nutritional decline may reflect poor local immune responses and influence the tolerance of, and compliance with treatment (385, 495).

To our knowledge this is the first report to link hypoalbuminaemia and response to neoadjuvant chemotherapy in a series of predominately oesophagogastric adenocarcinomas. A recent study has found NLR to be a predictor of chemosensitivity in squamous cell carcinoma of the oesophagus (496). These studies are in keeping with

previous reports in other cancer types that have found malnutrition and hypoalbuminaemia to be associated with lack of response to chemotherapy as well as worse short term and long term outcomes after both chemotherapy and surgery (497-499). Traditionally, researchers have sought to find factors associated with survival after surgery or response to chemotherapy as two separate entities. This study suggests that the host response to tumour in terms of inflammation and blood borne markers of immunonutritional status may determine both response to chemotherapy and survival and therefore interventions to improve outcomes should target patients throughout the treatment pathway. In addition, it may be beneficial to assess these markers during neoadjuvant treatment as this may add to the ability to detect those patients who are likely to respond to treatment and therefore gain survival benefit (500). This is important because a definite response to neoadjuvant chemotherapy is only observed in a proportion of patients, in this cohort 19% (TRG 1-3), and the remainder may have potentially curative surgery delayed unnecessarily.

The use of serum albumin in this context is not without controversy and many question its role in assessing nutritional status. The use of non-biochemical nutritional screening tools such as Malnutrition Universal Screening Tool (MUST) (501) or Nutritional Risk Screening 2002 (NRS-2002) (502) may provide more sensitive nutritional assessment of the patients in this study. MUST has only recently been introduced into the routine assessment of our patients and so did not form part of this study. However, serum albumin is one of the most commonly used methods to assess nutritional status and a recent systematic review of the epidemiological literature has confirmed serum albumin as a prognostic indicator of cancer survival (503). Albumin concentrations have also been shown, in cancer patients, to correlate with ideal body weight, percent weight loss, and percent predicted total body potassium (body cell mass) (504). Several processes control plasma albumin concentration including rate of synthesis, the fractional catabolic rate and total body albumin distribution. Furthermore, the rate of albumin synthesis is influenced by both nutrition and inflammation and it has been suggested that it is primarily the effect of inflammation on albumin levels that is responsible for much of the morbidity and mortality associated with hypoalbuminaemia (505).

The retrospective nature of this type of analysis is subject to shortcomings including data collection and selection bias. Missing data may have a significant impact on the accuracy of a test but is rarely reported making it difficult to interpret previous series with confidence and data synthesis unreliable. In this series all patients had preoperative NLR and 96% of patients analysed had serum albumin recorded. Unfortunately CRP was not available in sufficient numbers in this cohort for comparison as currently it is not our routine practise. Previous studies have highlighted the prognostic value of CRP or CRP based scores such as GPS (506), combining NLR with CRP and strong associations of CRP with NLR (507). However, the lack of available CRP values

highlights that currently markers of systemic inflammation have not entered everyday practice, despite the growing evidence for their clinical utility, and perhaps they should.

A critical deficiency in the current literature is the lack of good quality clinical trials demonstrating that anti-inflammatory or nutritional interventions decrease the excess mortality in cancer. The majority of trials have either been conducted on a heterogenous patient cohort, or have been designed to assess the impact of immunonutrition in the immediate perioperative period (+/-7days) (508-511). However, recent evidence suggests that marine n-3 fatty acids possess a potent anti-inflammatory effect (512) and it is reasonable to expect that supplementation with a fish oil based product may restore the balance of pro and anti inflammatory mediators in upper GI cancer. Our evidence suggests that future clinical studies should examine the role of modulators of immunonutritional status during the administration of neoadjuvant therapy and in the perioperative period, and that these studies will need to be sufficiently powered to demonstrate modest changes in outcome. These could include immunonutrition or specific (antibodies to TNF-α and IL-6) (500) and non-specific anti-inflammatory interventions, such as aspirin which is currently being investigated in the AspECT trial (513).

In summary, this study has confirmed that biochemical markers of nutritional status and the systemic inflammatory response are independent predictors of survival in patients undergoing radical treatment for oesophagogastric cancer. Preoperative nutritional status and increased systemic inflammation are potentially modifiable interacting risk factors. For instance, it may be possible to target treatments to patients during neo-adjuvant chemotherapy or during the perioperative period (514-516). A recent review of immunonutrition in patients undergoing oesophageal cancer resection has called for well designed randomized controlled trials to assess the potential benefits of immunonutrition targeted to the nutritionally compromised (517). Whilst these strategies will require prospective trials over long periods, the more immediate impact of this study maybe to better risk-stratify patients by incorporating available biochemical markers of systemic inflammation and nutritional status into current preoperative risk assessment tools.

8. Chapter 8: Tumour Infiltrating Lymphocytes Associate with Improved Survival in Oesophageal Adenocarcinoma

8.1. Introduction

Virchow first described the association of lymphocyte infiltration with solid tumours in 1863 (317). Immune responses against malignant cells, whether systemic or within the tumour microenvironment, are increasingly implicated as a determining factor in tumour progression (487). The most comprehensive studies, to establish this hallmark of cancer (221), have been in colorectal tumours (227). Specific tumour infiltrating lymphocyte (TIL) subsets communicate and function to affect tumour growth and the balance of these effects leads to either tumour regression or tumour-promotion. An anti-tumour effect is mediated by the combination of cancer cell lysis and the production of cytotoxic cytokines, supported by CD4+ T helper cells. In contrast, T regulatory cells, Fox3p+ T cells, act to suppress the immune response of other cells and so affect the inflammatory process indirectly (487). Therefore, understanding the impact of specific subsets of immune cells that infiltrate tumours is important for making rational decisions in the development of targeted therapies.

Oesophageal adenocarcinoma (OAC) is becoming increasingly common in the Western world and despite a variety of strategies to improve outcome (518), survival remains poor at 10-15% at 5 years (21). Radical treatment with curative intent includes neoadjuvant therapy, and oesophagogastrectomy. Neoadjuvant chemotherapy (NAC) delivers improved survival in a small percentage of patients, in whose tumours there is a significant pathological response to treatment (3). However, approximately 2/3 of patients present with advanced, incurable disease at diagnosis. Biological therapies such as growth factor blockers, poly ADP ribose polymerase (PARP) inhibitors, vaccines and monoclonal antibodies are being evaluated and are yet to enter routine clinical practice (519). The rapid rise in incidence of OAC in the West means that novel therapies are urgently required, either as single agents or for use in combination with conventional treatments.

In particular, the ability of CD8+ lymphocytes to recognise tumour antigens has been well documented (520), and tumour regression is observed when tumour reactive T lymphocytes invade cancers (521). Before considering immune therapies as a potential treatment in OAC it is important to understand if immune cells are present in OAC, which sub-sets of cells are observed and whether or not they have prognostic significance.

The effects of tumour infiltrating lymphocytes (TILs) have been studied in a range of solid tumours and high TIL density correlates with better survival (reviewed in (522)). A few initial studies have examined the association of TILs in OAC with prognosis (325, 386). These studies have provided conflicting results and no study has assessed TIL density in resected tumours after neoadjuvant chemotherapy and whether or not there is a link to outcome. Schumacher et al observed intratumoural CD8+ T-cell infiltration to correlate with improved survival in a small heterogeneous cohort of OAC (n=37) and

oesophageal squamous cell carcinoma (OSCC) (n=33) patients (230). Additional studies have shown that the overall grade of TIL density at the invasive margin, correlates with improved survival in patients with gastroesophageal cancer (386). In contrast, Zingg et al did not find any independent associations between differing TIL subsets and survival in OAC patients who received multimodal therapy with either neoadjuvant chemoradiotherapy or surgery alone. However they did demonstrate in univariate analyses that particular subtypes of TIL conferred a better survival when dichotomised at median counts (CD3+, CD8+, Fork head box P3+ (FOXP3+), CD8+:CD4+) (344). Therefore, the prognostic value of TILs in OAC remains to be established and the composition of TIL density following neoadjuvant chemotherapy in OAC is yet to be assessed.

In this study, we assessed the immune infiltrate in a large contemporary cohort of OAC (n>120) to identify association with survival and clinicopathological disease characteristics. Specifically, we examined TILs, as their presence would suggest that active immunotherapy might be attractive in this condition. A link between pre-existing immunity might then also allow us to stratify patients into groups, more or less likely to benefit from immunmodulation such as checkpoint blockade, which allows a realease of pre-existing immune responses for clinical benefit. . In addition we analyse TIL frequencies following neoadjuvant chemotherapy and assess the relationship of TILs and disease outcome.

Method

8.1.1. Patients

For this retrospective study, a prospectively collected database of consecutive patients undergoing oesophagogastric resection for OAC treated at University Hospital Southampton NHS Foundation Trust (UHSFT) between January 2005 and December 2010 was reviewed. All patients followed a contemporary treatment pathway with description of general parameters included and analysed included in chapter 2 (Chapter 2, 2.2.3). Patients excluded from the study included those with a complete pathological response to the tumour (TRG1), inpatient mortality and a lack of available histopathological tissue.

8.1.2. Immunohistochemistry

4 µm sections of TMA blocks were used to assess the immune infiltrate. The ABC method of immunoperoxidase labelling was used to identify expression of antigens using either a manual or automated protocol (Chapter 2, 2.3.6). Within each core, only tumour cells or representative areas of normal oesophagus were scored. The incorporation of 3 cores on the TMAs for each patient and area of interest ensured representative areas were available for scoring (Chapter 2, 2.3.6.10.).

All sections were digitally image captured to enable sections to be scored under the supervision of experienced pathologists. The majority of published work studying immune infiltration of tumours used quantitative scoring. 3 high powered fields (×20 magnification) with the highest density of the marker of interest were scored, with a mean taken, providing a total scoring area of 0.1944 mm^2. This is in keeping with previous studies assessing the number of intra-tumoral immune cells. Immune cells (CD3+, CD4+, CD8+, CD68+ and FOXP3+) studied are summarised in chapter 1 (Table 1-4) with antibodies used documented in chapter 2 (Table 2-12).

Following scoring, some expression data needed grouping to facilitate statistical analysis. This was done using justifiable cut-offs (high-low/either side of the median) (Chapter 2, 2.3.6.9.1.).

8.1.3. Statistical analysis

Descriptive data are represented as median and range unless indicated with Kruskal-Wallis, Mann Whitney U and Pearson's chi-squared test used as appropriate for comparison. Kaplan-Meier, univariate and multivariate cox logistic regression modelling were used to assess the relationship between immune infiltration with CSS, DFS and response to neoadjuvant chemotherapy as outcomes. All factors that showed statistical significance on univariate analysis were entered to derive the final model using the backward stepwise likelihood ratio method. CSS and DFS curves of the patients were plotted by using the Kaplan-Meier method and analysed using the Log-rank test. Stratified analyses were performed based on receipt of neoadjuvant chemotherapy and pathological response to chemotherapy. A p value <0.05 was considered statistically significant for all tests. Statistical analysis was performed with SPSS® version 21 (SPSS, Chicago, Illinois, USA).

8.2. Results

8.2.1. Study patients

A total of 128 patients were included in the study with median follow up of 3.5 years (median follow up: 3.485 years 95% CI: 2.629-4.342). The study population had a median age, at operation, of 68 years (range, 46–85 years) and were predominately male (88%). The majority had pathologically advanced staged disease, pT/ypT3 (51%) and node positive disease (54%), and were located either at the oesophagogastric junction (64%) or lower oesophagus (36%).

76 (59.4%) patients received multimodal therapy, neoadjuvant chemotherapy and surgery, with 52 (40.6%) patients proceeding directly to surgery alone. Of the patients that received multimodal therapy, 11 (15%) patients had a significant local tumoural response (TRG 2) and 30 (40%) patients had a significant lymph node response (lymph node downstaging: cN1 to ypN0) to neoadjuvant chemotherapy. Detailed patient

characteristics and clinical and pathological outcomes are summarised in supplemental Table 8-1.

8.2.2. The relationship of intratumoural infiltrating lymphocytes and clinicopathological characteristics

Representative TIL staining is shown in supplemental Figure 8-5. The relationship of patient and tumour characteristics to TILs is presented in supplemental Table 8-2 and 8-3 respectively.

A reduced TIL level, across all subsets, was significantly associated with increasing stage of disease (T and N stage) and with vascular, lymphatic and perineural invasion (Table 8-3).

Higher TILs counts were observed in female compared to male patients (Table 8-2). This was statistically significant for CD4+ (p=0.036), CD8+ (p=0.037), and FOXP3+ (p=0.045) cells. No statistically significant differences were found regarding premorbid status (Performance status, ASA grade, Age or Smoking status, Table 8-2).

The relationship of TILs were not statistically different between patients treated with multimodal therapy, neoadjuvant chemotherapy and surgery, or surgery alone and is presented in Table 8-2 and 8-4. The distribution of TILs was positively skewed with a heavier left than right tail. The distribution of CD8+ TILs is shown as an example in Figure 1.

The number of TILs positively correlated with each other for all subtypes (range of correlation coefficient = 0.677-0.905; p<0.001) and are detailed in Table 8-5.

8.2.3. Prognostic Significance of intratumoural infiltrating lymphocytes

Median follow up was 3.5 years with the results of the Cox proportional hazard model for predictors of CSS and DFS shown in Table 8-6 and Table 8-7 respectively. Multivariate analysis identified independent prognostic factors for improved CSS to be reduced pathological N stage (p<0.0001), increased CD8+ TILS (p=0.003) and completeness of resection (p<0.0001). The hazard ratio for increasing CD8+ TILs was 0.847 (95% CI: 0.760-0.944). Dichotimization of the TIL data either at the medians or into tertiles (None, Low < Median, or High ≥ Median TIL levels) allowed Kaplan-Meier survival analysis are presented in Figure 8-2 showing increasing levels were associated with improved CSS. CD8+ TILS ≥ 5 were associated with better CSS (CD8+ TILs ≥ 5: mean (median not yet reached) CSS 5.1 years, 95% CI: 4.4-5.8, vs. CD8+ TILs <5: median CSS 1.9 years, 95% CI: 1.3-2.4, p<0.0001).

Multivariate analysis identified independent prognostic factors for reduced DFS as reduced CD8+ TILs (p<0.0001), pathological N stage (p<0.0001), incompleteness of resection (p=0.001) and multimodal treatment (p=0.015). The hazard ratio for increasing CD8+ TILs was 0.894 (95% CI: 0.844-0.948). Kaplan-Meier survival analysis is presented in Figure 8-3 showing that increasing levels of TILs were associated with improved DFS.

CD8+ TILS ≥ 5 were associated with better DFS (CD8+ TILs ≥ 5: mean (median not yet reached) DFS 4.7 years, 95% CI: 3.9-5.4, vs. CD8+ TILs <5: median DFS 1.2 years, 95% CI: 0.9-1.5, p<0.0001).

8.2.4. The relationship of intratumoural infiltrating lymphocytes and response to neoadjuvant chemotherapy

Increasing levels of TILs were significantly associated with improved pathological response to neoadjuvant chemotherapy, both in the primary tumour and the lymph nodes, as detailed in Tables 8-8, 8-9, Tables 8-10, 8-11 and Figure 8-4. There was increased CD3+ (p=0.007), CD4+ (p=0.025), and CD8+ (p=0.002) TIL frequencies in the tumour of patients who had LN downstaging after neoadjuvant chemotherapy. Increased TIL frequencies were also seen in patients who had a significant pathological response to neoadjuvant chemotherapy (TRG2) although this did not reach statistical significance.

On multivariate analysis CD4+ TILs (p=0.017) and CD8+ TILs (p=0.005) were associated with significant tumour response (TRG) and lymph node downstaging respectively shown in table 8-9 and table 8-11.

Table 8-1: Clinicopathological characteristics of patient cohort of which formed TMAs

		n=128
Operation Age*		67.77 (45.48-85.41)
Sex ratio (M:F)		112:16
ASA	**1**	13 (10.2)
	2	93 (72.4)
	3	22 (17.3)
Tumour site	**Lower 1/3**	46 (35.9)
	OGJ – S1	25 (19.5)
	OGJ – S2	26 (20.3)
	OGJ – S3	31 (24.2)
Type	**AC**	128 (100)
pT or ypT	**T1**	29 (22.7)
	T2	30 (23.4)
	T3	65 (50.8)
	T4	4 (3.1)
pN or ypN	**N0**	59 (46.1)
	N1	28 (21.9)
	N2	21 (16.4)
	N3	20 (15.6)
pM or ypM	**M0**	125 (97.7)
	M1	3 (2.3)
Resection clearance (R0)		104 (81.3)
Vascular Invasion		51 (39.8)
Lymphatic Invasion		21 (16.4)
Perineural Invasion		17 (13.3)
Neoadjuvant chemotherapy		76 (59.4)
Neoadjuvant regime	**ECX**	64 (50)
	EOX	10 (7.8)
	ECF	2 (1.6)
Tumour regression grade	**1**	0 (0)
	2	11 (8.6)
	3	10 (7.8)
	4	29 (22.7)
	5	26 (20.3)
	Not assessed	0 (0)
	Surgery only	52 (40.6)
Nodal downstaging		30/76 (39.5)

Values in parentheses are percentages unless indicated.

*Values in parentheses are range

ASA - American Society of Anesthesiologists physical status classification system

Table 8-2: Relationship of Intratumoural TILs and OAC patient characteristics

		CD3+	CD4+	CD8+	FOXP3+
Age/ years	*p*-value	0.521	0.347	0.529	0.308
	<75	11.00 (0.00-27.00)	3.30 (0.00-27.00)	4.85 (0.00-37.70)	1.30 (0.00-21.00)
	≥75	14.00 (0.00-77.30)	5.00 (0.00-43.00)	5.85 (0.00-38.00)	2.00 (0.00-14.70)
Sex	*p*-value	0.059	**0.036**	**0.037**	**0.045**
	Male	11.00 (0.00-74.00)	**3.30 (0.00-27.00**	**4.70 (0.00-38.00)**	**1.15 (0.00-21.00)**
	Female	22.20 (0.00-77.30)	**7.85 (0.00-43.00)**	**9.00(0.00-25.70)**	**2.50 (0.00-10.70)**
ASA	*p*-value	0.338	0.553	0.697	0.173
	1	14.30 (0.00-43.30)	2.30 (0.00-22.00)	6.00 (0.00-16.70)	1.00 (0.00-21.00)
	2	12.65 (0.00-77.30)	4.30 (0.00-43.00)	5.50 (0.00-37.70)	2.00 (0.00-13.30)
	3	4.65 (0.00-77.30)	1.00 (0.00-16.70)	1.35 (0.00-38.00)	0.00 (0.00-11.30)
Performance Status	*p*-value	0.944	0.664	0.893	0.818
0		12.30 (0.00-71.00)	2.30 (0.00-22.00)	5.00 (0.00-29.00)	1.70 (0.00-21.00)
1		10.85 (0.00-77.30)	4.00 (0.00-19.30)	5.00 (0.00-38.00)	1.00 (0.00-13.30)
2		14.85 (0.00-72.00)	4.50 (0.00-27.00)	6.35 (0.00-32.70)	1.30 (0.00-21.00)
Smoker Preop	*p*-value	0.110	0.088	0.174	0.147
Yes		16.00 (0.00-74.00)	4.50 (0.00-27.00)	6.70 (0.00-38.00)	2.00 (0.00-21.00)
No		10.70 (0.00-72.00)	3.30 (0.00-22.00)	4.70 (0.00-32.70)	1.00 (0.00-13.30)
Treatment	*p*-value	0.945	0.949	0.996	0.864
Surgery only		12.00 (0.00-77.30)	3.85 (0.00-43.00)	5.85 (0.00-38.00)	1.30 (0.00-21.00)
Neoadjuvant Rx + Surgery		12.00 (0.00-64.00)	3.70 (0.00-27.00)	5.00 (0.00-20.70)	1.30 (0.00-11.30)

Values median with range in parentheses

Table 8-3: Relationship of Intratumoural TILs and OAC tumour characteristics

		CD3	CD4	CD8	FOXP3+
ypT or pT	*p*-value	**0.040**	**0.014**	**0.020**	**0.084**
	T1	**15.00 (0.00-77.30)**	**7.00 (0.00-43.00)**	**7.00 (0.00-25.70)**	**2.00 (0.00-21.00)**
	T2	**12.85 (0.00-72.00)**	**4.85 (0.00-27.00)**	**6.50 (0.00-37.70)**	**2.00 (0.00-13.30)**
	T3	**11.70 (0.00-74.00)**	**2.30 (0.00-20.00)**	**4.70 (0.00-38.00)**	**1.00 (0.00-14.70)**
	T4	**0.00 (0.00-12.00)**	**0.00 (0.00-4.70)**	**0.00 (0.00-3.70)**	**0.00 (0.00-6.70)**
ypN or pN	*p*-value	**<0.0001**	**<0.0001**	**<0.0001**	**0.008**
	N0	**16.00 (0.00-77.30)**	**6.00 (0.00-43.00)**	**8.30 (0.00-37.70)**	**2.00 (0.00-21.00)**
	N1	**13.35 (0.00-29.30)**	**3.65 (0.00-10.70)**	**5.20 (0.00-15.00)**	**1.65 (0.00-9.30)**
	N2	**0.00 (0.00-49.30)**	**0.00 (0.00-10.70)**	**0.00 (0.00-29.30)**	**0.00 (0.00-9.30)**
	N3	**7.85 (0.00-74.00)**	**2.00 (0.00-20.00)**	**2.00 (0.00-38.00)**	**0.00 (0.00-14.70)**
Resection clearance	*p*-value	**0.090**	**0.040**	0.088	**0.017**
	R1	**8.35 (0.00-64.00)**	**1.15 (0.00-20.00)**	1.50 (0.00-18.70)	**0.00 (0.00-10.00)**
	R0	**13.35 (0.00-77.30)**	**4.00 (0.0043.00)**	5.85 (0.00-38.00)	**1.70 (0.00-21.00)**
Vascular Invasion	*p*-value	**0.048**	**0.030**	**0.011**	**0.027**
	Yes	**10.70 (0.00-64.00)**	**1.70 (0.00-27.00)**	**4.30 (0.00-29.30)**	**1.00 (0.00-9.30)**
	No	**13.70 (0.00-77.30)**	**4.30 (0.00-43.00)**	**6.70 (0.00-38.00)**	**1.70 (0.00-9.30)**
Lymphatic Invasion	*p*-value	**0.022**	**0.038**	**0.037**	**0.182**
	Yes	**3.30 (0.00-43.40)**	**1.00 (0.00-27.00)**	**1.30 (0.00-15.00)**	**0.00 (0.00-9.30)**
	No	**13.70 (0.00-77.30)**	**4.30 (0.00-43.00)**	**5.70 (0.00-38.00)**	**1.30 (0.00-21.00)**
Perineural Invasion	*p*-value	**0.016**	**0.001**	**0.025**	**0.033**
	Yes	**0.00 (0.00-36.00)**	**0.00 (0.00-11.00)**	**0.00 (0.00-14.00)**	**0.00 (0.00-8.00)**
	No	**12.30 (0.00-77.30)**	**4.00 (0.00-43.00)**	**5.70 (0.00-38.00)**	**1.30 (0.00-21.00)**

Values median with range in parentheses

Table 8-4: Descriptive statistics for number of TILs in OAC stratified by treatment modality

		CD3+ TILs	CD4+ TILs	CD8+ TILs	FOXP3+ TILs
Surgery Only	**Mean***	19.49	5.69	8.13	3.42
	Median*	12.00	3.85	5.85	1.30
	Variance	525.20	55.87	105.10	23.13
	Range*	0.0-77.3	0.0-43.0	0.0-38.0	0.0-21.0
	Skewness	1.224	2.771	1.572	1.689
Neoadjuvant Rx + Surgery	**Mean***	15.57	4.94	6.11	3.42
	Median*	12.00	3.70	5.00	1.30
	Variance	206.54	31.98	28.95	7.91
	Range*	0.0-64.0	0.0-27.0	0.0-20.7	0.0-11.3
	Skewness	0.877	1.715	0.679	1.291
Total	**Mean***	17.16	5.25	6.93	2.81
	Median*	12.00	3.70	5.00	1.30
	Variance	336.61	41.46	60.30	14.21
	Range*	0.0-77.3	0.0-43.0	0.0-38.0	0.0-21.0
	Skewness	1.329	2.428	1.790	1.900

*Data are cells counted per field; No statistically significant difference by treatment modality

Table 8-5: Correlation between subtypes of intratumoural TILs

		FOXP3+	CD8+	CD4+
CD3+	*Correlation coefficient*	**0.791**	**0.905**	**0.836**
	p-value	**<0.0001**	**<0.0001**	**<0.0001**
CD4+	*Correlation coefficient*	**0.677**	**0.710**	
	p-value	**<0.0001**	**<0.0001**	
CD8+	*Correlation coefficient*	**0.695**		
	p-value	**<0.0001**		

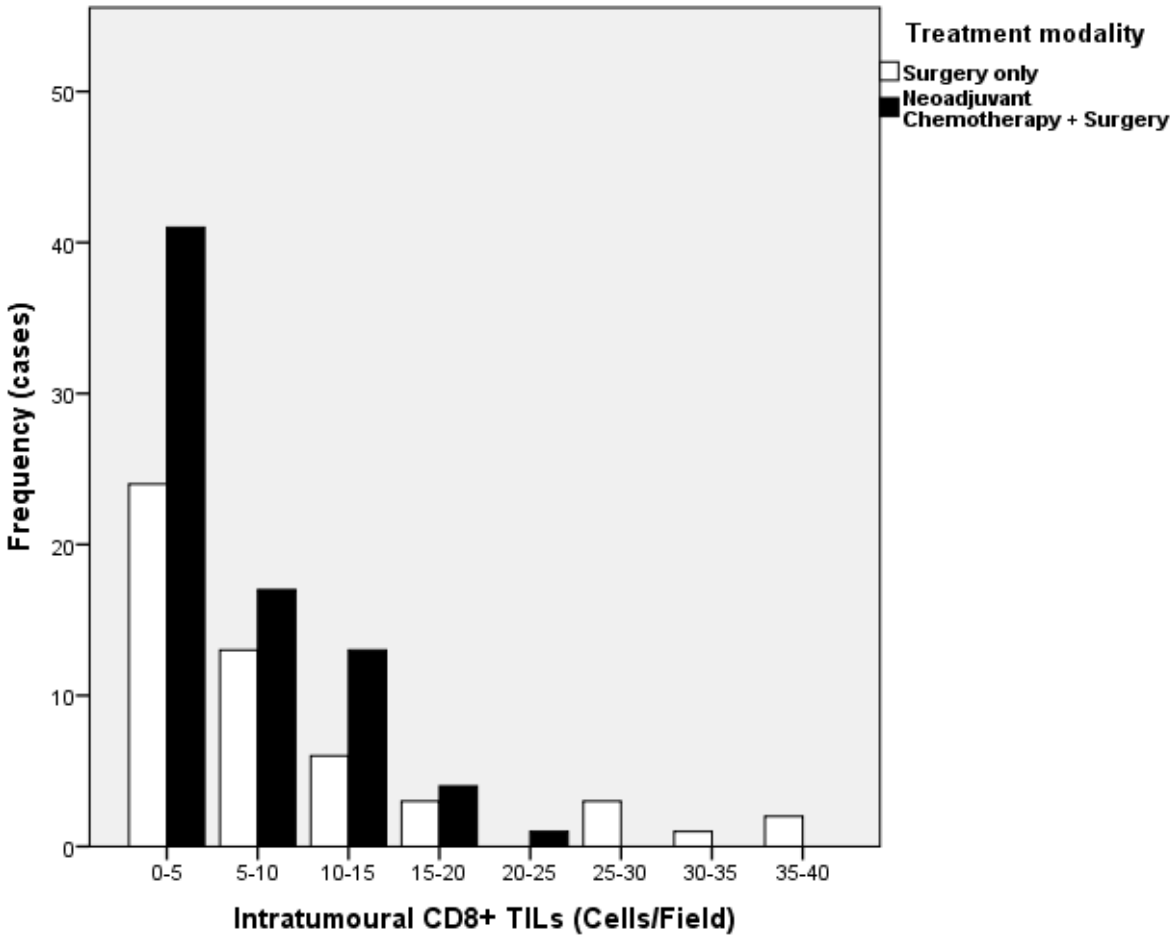


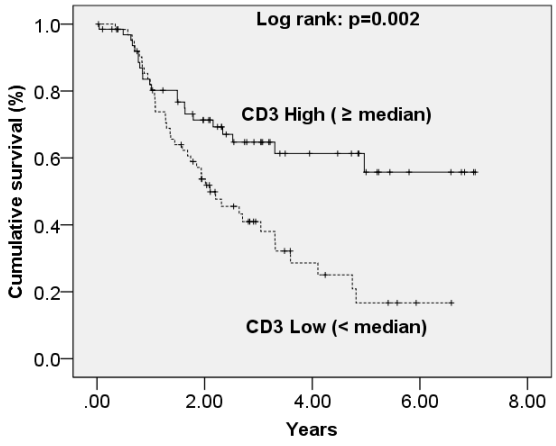
Figure 8-1: Frequency distribution of CD8+ TILs in OAC by treatment modality

Table 8-6: Univariate and multivariate Cox regression analyses of patient and tumour factors with OAC specific Survival

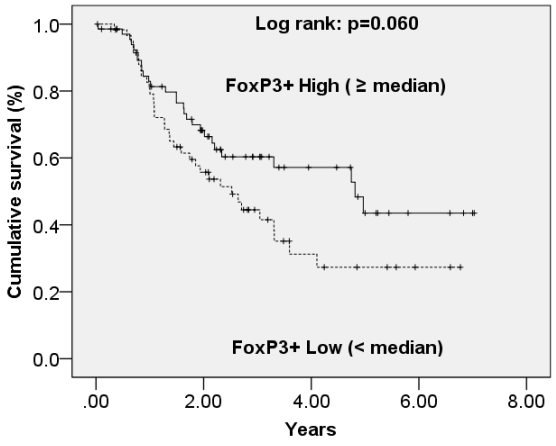
		Univariate			Multivariate		
		HR	95%CI	P value	HR	95% CI	*p* value
Patient factors							
Age		0.987	0.961-1.014	0.348			
Sex	Female	1	Ref				
	Male	1.088	0.495-2.389	0.834			
ASA	1	1	Ref				
	2	1.670	0.659-4.231	0.280			
	3	1.398	0.485-4.031	0.535			
Performance status	0	1	Ref				
	1	0.867	0.475-1.580	0.640			
	2	0.606	0.199-1.843	0.377			
Preoperative smoker	No	1	Ref				
	Yes	0.835	0.491-1.421	0.507			
Neoadjuvant Rx	No	1	Ref		1	Ref	
	Yes	1.723	1.011-2.937	**0.046**	1.703	0.977-2.971	0.061
Immunohistochemistry							
CD3		0.966	0.947-0.985	**0.001**	1.035	0.997-1.075	0.075
CD4		0.927	0.874-0.982	**0.010**			
CD8		0.895	0.849-0.944	**<0.0001**	0.847	0.760-0.944	**0.003**
FOXP3+		0.879	0.801-0.966	**0.007**			
Tumour factors							
ypT or pT stage	1	1	Ref				
	2	2.450	0.947-6.338	0.065			
	3	4.480	1.872-10.720	**0.001**			
	4	16.094	4.385-59.071	**<0.0001**			
ypN or pN stage	0	1	Ref		1	Ref	
	1	4.036	2.033-8.014	**<0.0001**	3.745	1.806-7.764	**<0.0001**
	2	6.063	2.889-12.723	**<0.0001**	3.476	1.515-7.974	**0.003**
	3	5.143	2.464-10.735	**<0.0001**	3.607	1.635-7.959	**0.001**
ypM or pM stage	0	1	Ref				
	1	4.546	1.402-14.746	**0.012**			
Vascular invasion	No	1	Ref				
	Yes	2.234	1.354-3.684	**0.002**			
Lymphatic Invasion	No	1	Ref				
	Yes	1.985	1.109-3.554	**0.021**			
Perineural Invasion	No	1	Ref				
	Yes	2.811	1.519-5.201	**0.001**			
Resection clearance	R0	1	Ref		1	Ref	
	R1	3.027	1.737-5.273	**<0.0001**	3.134	1.699-5.783	**<0.0001**

Table 8-7: Univariate and multivariate Cox regression analyses of patient and tumour factors with OAC disease free survival

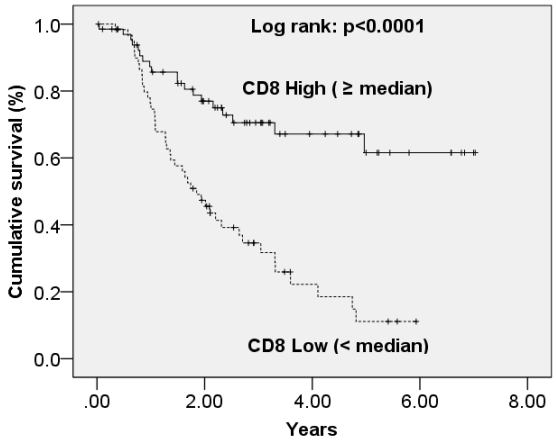
		Univariate			Multivariate		
		HR	95%CI	P value	HR	95% CI	*p* value
Patient factors							
Age		0.978	0.953-1.003	0.085			
Sex	Female	1	Ref				
	Male	1.305	0.596-2.855	0.505			
ASA	1	1	Ref				
	2	1.571	0.672-3.674	0.297			
	3	1.028	0.373-2.833	0.957			
Performance status	0	1	Ref				
	1	0.771	0.442-1.344	0.359			
	2	0.644	0.237-1.749	0.388			
Preoperative smoker	No	1	Ref				
	Yes	0.915	0.555-1.509	0.727			
Neoadjuvant Rx	No	1	Ref		1	Ref	
	Yes	1.996	1.183-3.367	**0.010**	1.998	1.145-3.487	**0.015**
Immunohistochemistry							
CD3		0.960	0.941-0.980	**<0.0001**			
CD4		0.895	0.842-0.951	**<0.0001**			
CD8		0.886	0.841-0.933	**<0.0001**	0.894	0.844-0.948	**<0.0001**
FOXP3+		0.885	0.811-0.965	**0.006**			
Tumour factors							
pT stage	1	1	Ref				
	2	4.325	1.610-11.618	0.004			
	3	6.180	2.424-15.758	**<0.0001**			
	4	15.913	4.178-60.604	**<0.0001**			
pN stage	0	1	Ref		1	Ref	
	1	5.796	2.988-11.243	**<0.0001**	5.178	2.630-	**<0.0001**
	2	7.899	3.822-16.324	**<0.0001**	3.584	1.639-7.840	**0.001**
	3	6.351	3.036-13.288	**<0.0001**	5.142	2.353-	**<0.0001**
pM stage	0	1	Ref				
	1	4.479	1.374-14.598	**0.013**			
Vascular invasion	No	1	Ref				
	Yes	2.671	1.657-4.307	**<0.0001**			
Lymphatic Invasion	No	1	Ref				
	Yes	2.099	1.213-3.635	**0.008**			
Perineural Invasion	No	1	Ref				
	Yes	2.450	1.309-4.584	**0.005**			
Resection clearance	R0	1	Ref		1	Ref	
	R1	2.563	1.492-4.405	**0.001**	2.600	1.448-4.666	**0.001**



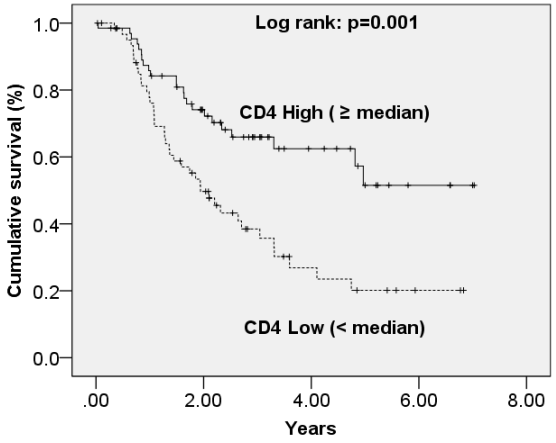
No. at risk				
Years	0	2	4	6
CD3 High	65	37	15	5
CD3 Low	63	29	8	1



No. at risk				
Years	0	2	4	6
Foxp3+ High	68	37	15	4
Foxp3+ Low	60	29	8	2

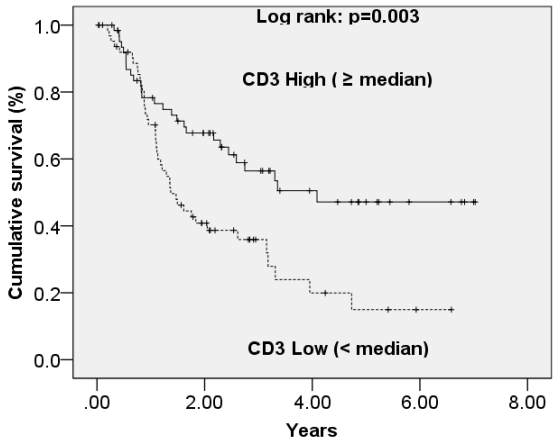


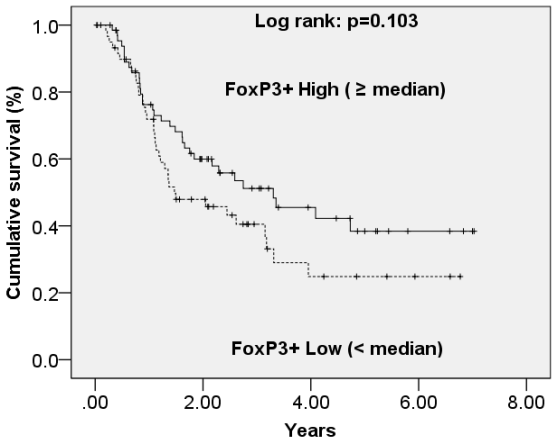
No. at risk				
Years	0	2	4	6
CD8 High	67	40	17	6
CD8 Low	61	26	6	0



No. at risk				
Years	0	2	4	6
CD4 High	66	39	15	4
CD4 Low	62	27	8	2

Figure 8-2: Kaplan-Meier curvess of cancer specific survival revealing prognostic significance of TILs in OAC



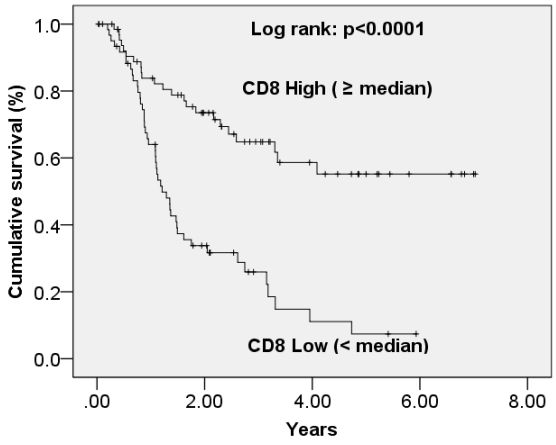


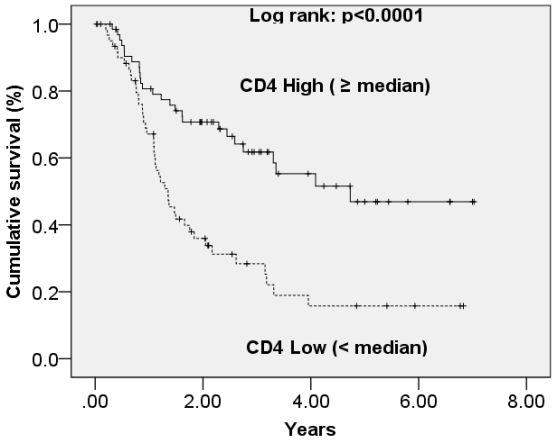
No. at risk

Years	0	2	4	6
CD3 High	65	35	15	5
CD3 Low	63	20	5	1

No. at risk

Years	0	2	4	6
FoxP3+ High	68	32	14	4
FoxP3+ Low	60	23	6	1





No. at risk

Years	0	2	4	6
CD8 High	67	38	17	6
CD8 Low	61	17	3	1

No. at risk

Years	0	2	4	6
CD4 High	66	37	15	4
CD4 Low	62	18	5	2

Figure 8-3: Kaplan-Meier curves of disease free survival revealing prognostic significance of TILs in OAC

Table 8-8: Clinical and pathological factors in OAC patients that received neoadjuvant chemotherapy (n=76) based on their response to chemotherapy using tumour regression grading (TRG)

		Responder (TRG 2) n=11	Non-responder (TRG 3-5) n=65	*p* value
Age*		66.58 (55.77-74.62)	62.46 (45.48-81.29)	0.253
Sex	**Male**	11 (100)	57 (87.7)	0.222
	Female	0 (0)	8	
Performance status	**0**	4 (36.4)	18 (27.7)	0.488
	1	7 (63.6)	45 (69.2)	
	2	0 (0)	2 (3.1)	
ASA	**1**	1 (9.1)	9 (14.1)	0.922
	2	9 (81.8)	48 (73.4)	
	3	1 (9.1)	8 (12.5)	
ypT	**1**	6 (54.5)	7 (10.8)	**<0.0001**
	2	4 (36.4)	17 (26.2)	
	3	1 (9.1)	39 (60.0)	
	4	0 (0)	2 (3.1)	
ypN	**0**	11 (100)	22 (33.8)	**<0.0001**
	1	0 (0)	15 (23.1)	
	2	0 (0)	15 (23.1)	
	3	0 (0)	13 (20)	
LN downstaged	**Yes**	11 (100)	19 (29.2)	**<0.0001**
	No	0 (0)	46 (70.8)	
Differentiation	**G1 – Well**	3 (27.3)	3 (4.6)	0.061
	G2 – Moderate	3 (27.3)	17 (26.2)	
	G3 – Poor	5 (45.5)	45 (69.2)	
Resection clearance	**R0**	11 (100)	51 978.5)	0.090
	R1	0 (0)	65 (21.5)	
Immunohistochemistry values*	**CD3**	16.00 (0.00-43.30)	12.00 (0.00-64.00)	0.160
	CD4	6.00 (0.00-27.00)	3.30 (0.00-20.00)	0.055
	CD8	8.30 (0.00-16.70)	4.70 (0.00-20.70)	0.065
	FOXP3+	2.70 (0.00-11.30)	1.30 (0.00-9.30)	0.202

Values in parentheses are percentages unless indicated. *Values in parentheses are range

Table 8-9: Univariate and multivariate analysis of immunohistochemical markers for response to chemotherapy (TRG 2) in OAC

	Univariate			Multivariate		
	HR	95%CI	*p* value	HR	95% CI	*p* value
CD3	0.967	0.927-1.009	0.119			
CD4	**0.885**	**0.800-0.978**	**0.017**	**0.885**	**0.800-0.978**	**0.017**
CD8	0.912	0.814-1.023	0.116			
FOXP3+	0.831	0.678-1.017	0.073			

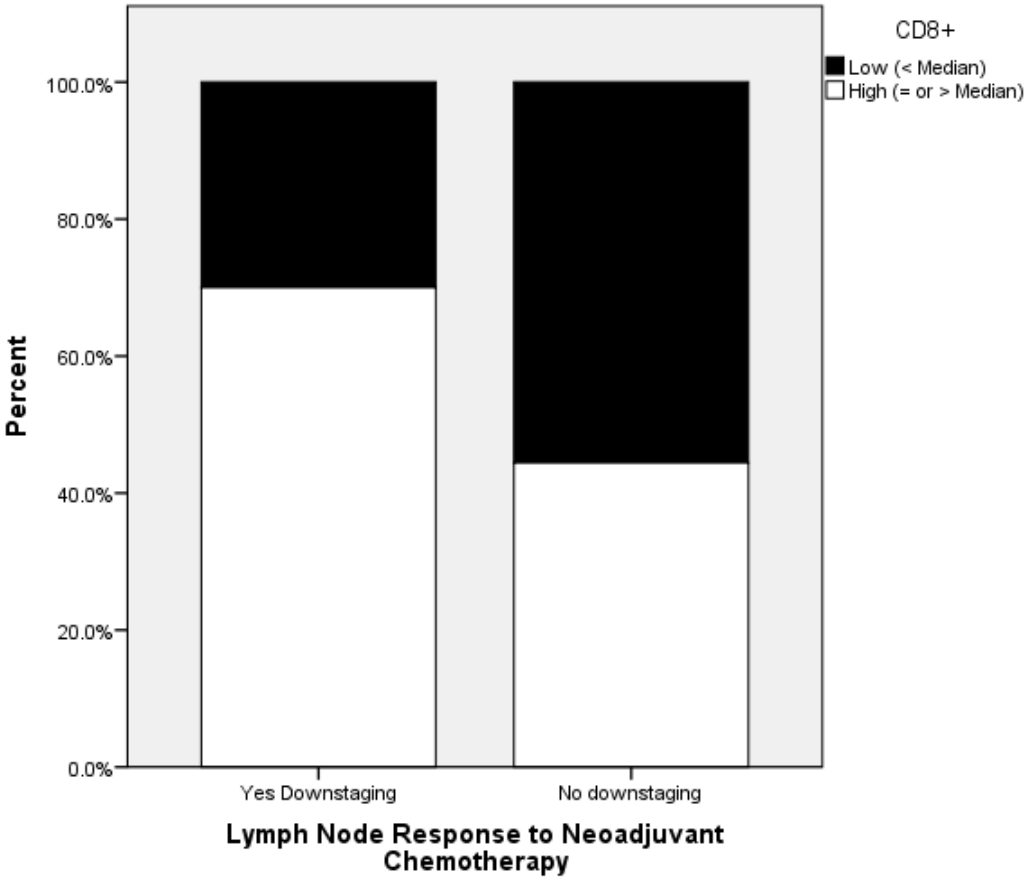
Table 8-10: Clinical and pathological factors in OAC patients that received chemotherapy (n=76) based on their response to neoadjuvant chemotherapy (LN downstaged)

		Responder n=30	Non-responder n=46	*p* value
Age*		63.68 (45.48-77.75)	62.62 (50.67-81.28)	0.941
Sex	**Male**	29 (96.7)	39 (84.8)	0.101
	Female	1 (3.3)	7 (15.2)	
Performance status	**0**	8 (26.7)	14 (30.4)	0.446
	1	20 (66.7)	32 (69.6)	
	2	2 (6.7)	0 (0)	
ASA	**1**	5 (16.7)	5 (11.1)	0.859
	2	20 (66.7)	37 (80.0)	
	3	5 (16.7)	4 (8.9)	
ypT	**1**	11 (36.7)	2 (4.3)	**<0.0001**
	2	10 (33.3)	11 (23.9)	
	3	9 (30.0)	31 (67.4	
	4	0 (0)	2 (4.3)	
ypN	**0**	30 (100)	3 (6.5)	**<0.0001**
	1	0 (0)	15 (32.6)	
	2	0 (0)	15 (32.6)	
	3	0 (0)	13 (28.3)	
Tumour Response	**Yes (TRG2)**	11 (36.7)	0 (0)	**<0.0001**
	No (TRG 3-5)	19 (63.3)	46 (100)	
Differentiation	**G1 – Well**	5 (16.7)	37 (80.4)	**0.036**
	G2 – Moderate	9 (30.0)	1 (2.2)	
	G3 – Poor	16 (53.3)	11 (23.9)	
Resection clearance	**R0**	2 (6.7)	12 (26.1)	**0.034**
	R1	28 (93.3)	34 (73.9)	
Immunohistochemistry	**CD3**	**15.15 (0.00-44.30)**	**11.20 (0.00-64.00)**	**0.007**
values*	**CD4**	**5.00 (0.00-27.00)**	**2.30 (0.00-20.00)**	**0.025**
	CD8	**8.65 (0.00-20.70)**	**4.50 (0.00-18.70)**	**0.002**
	FOXP3+	1.85 (0.00-11.30)	1.00 (0.00-9.30)	0.074

Values in parentheses are percentages unless indicated. *Values in parentheses are range

Table 8-11: Univariate and multivariate analysis of immunohistochemical markers for response to neoadjuvant chemotherapy (LN downstaged) in OAC

	Univariate			Multivariate		
	HR	95%CI	*p* value	HR	95% CI	*p* value
CD3	**0.957**	**0.924-0.992**	**0.015**			
CD4	0.916	0.838-1.001	0.052			
CD8	**0.869**	**0.789-0.958**	**0.005**	**0.869**	**0.789-0.958**	**0.005**
CD68	**0.833**	**0.722-0.961**	**0.012**			
FOXP3+	0.868	0.734-1.026	0.097			



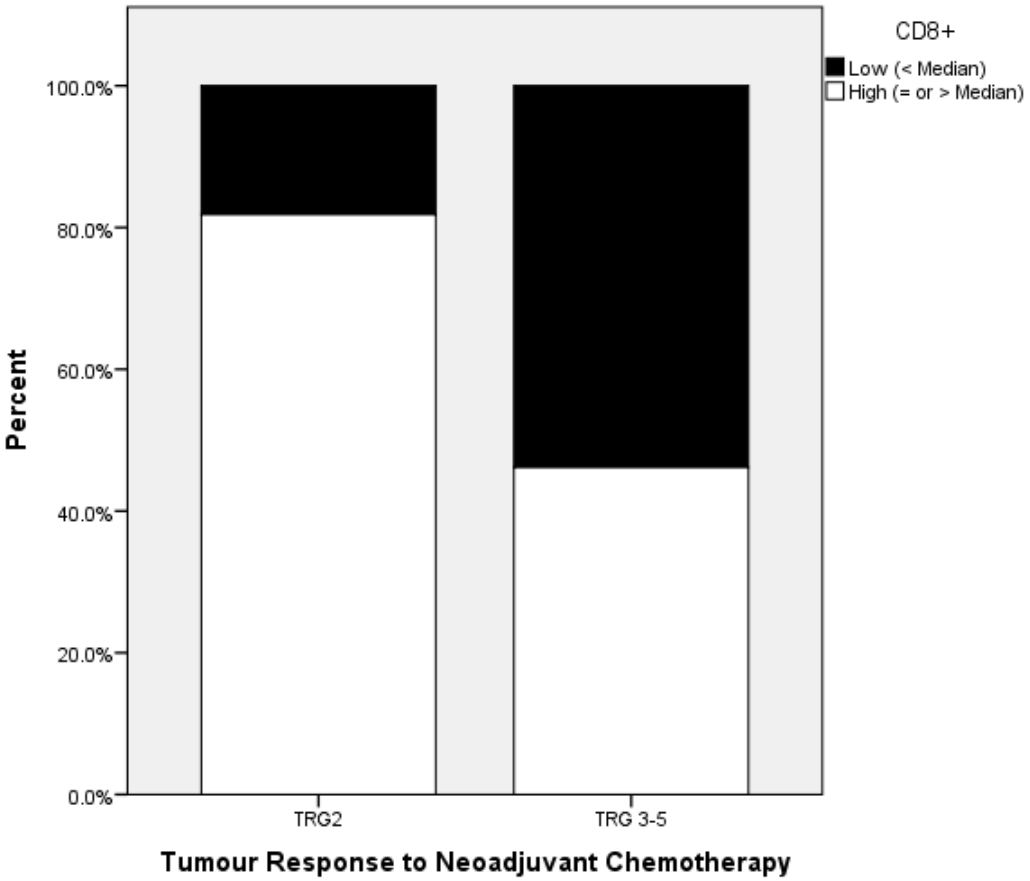


Figure 8-4: CD8+ immune infiltrate in OAC assessed by tumour response to chemotherapy

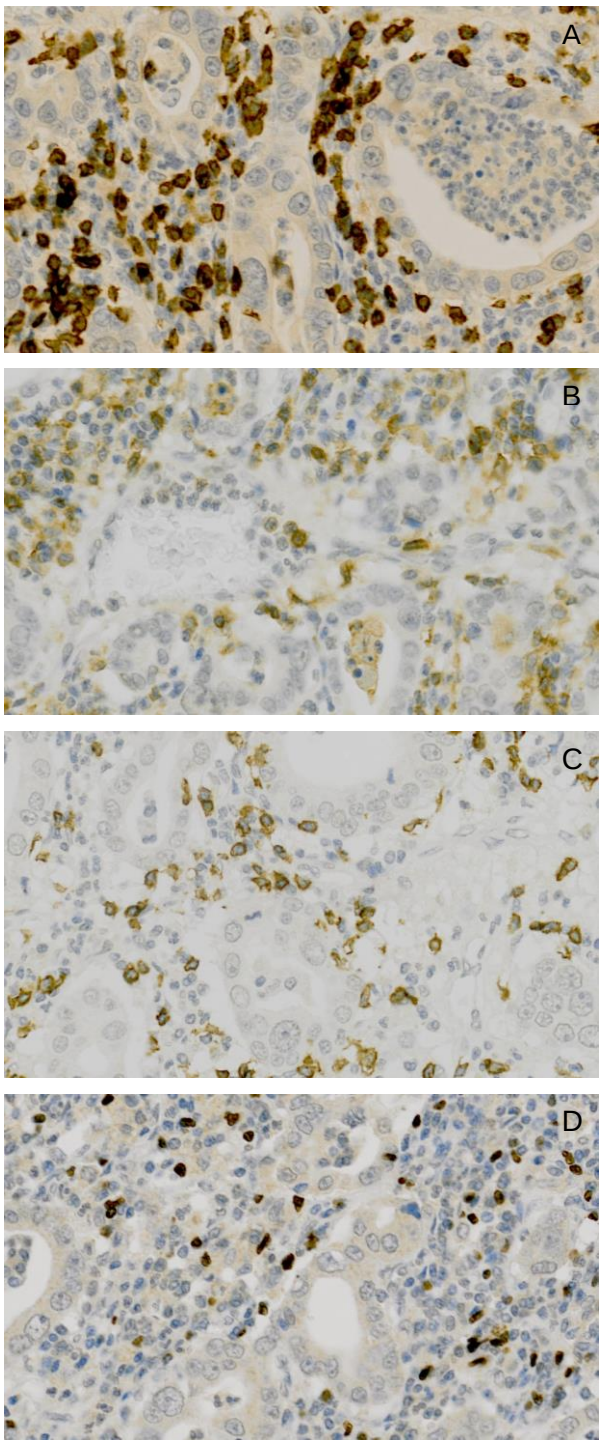


Figure 8-5: Tumour infiltrating lymphocytes CD3+ (A), CD4+ (B), CD8+ (C), FOXP3+ (D)

8.3. Discussion

In this study we have analysed the level of TILs in a large homogeneous cohort of oesophagogastric cancer patients following radical treatment with curative intent. We obtained three major findings: firstly, we have shown associations between pathological stage of disease and TIL density and confirmed the independent association of particular TIL subsets and survival; secondly we have shown significant correlation between TIL subtypes in OAC, and finally we found patients with a significantly increased pathological response to neoadjuvant chemotherapy had higher levels of TILs in their resected tumour, most notably with CD4+ and CD8+ TILs for local tumour regression and lymph node response respectively.

Of the TIL subsets analysed, CD8+ T-cells had the most significant independent association for both CSS (p=0.003) and DFS (p<0.0001). This has been previously suggested to be important in OAC, however independent association has not been universally verified (230, 344, 386). Zingg el al identified on univariate analysis a number of TILs as significant predictive factors however these were not independent factors when taking into account the stage of disease (344). In contrast Schumacher et al identified CD8+ TILs to be a significant predictive factor of survival independent of disease stage (230). The reasons for this discrepancy of findings between studies may relate to the clinicopathological factors used to build the multivariate statistical model, the location of TILs counted and the cut-off values used to dichotomise the TIL frequencies into high and low groups. In our large and homogeneous cohort we choose to evaluate TIL number as a continuous variable, counted intratumoural TILs and entered a comprehensive number of known clinicopathological predictors of survival into the univariate and subsequent multivariate statistical models. We believe this to be the most robust analysis of the association of TILs with survival on OAC that has been conducted to date.

In addition to the observed effects TILs have on disease recurrence, this study demonstrates that TILs play a role at different stages of disease. The significant association observed between TILs with T as well as N stage support the possibility that intratumoural T cells prevent tumour progression throughout the disease process. These findings are supported by previous smaller studies showing correlation with low TILs and higher stage of disease (230, 344).

A novel observation is that the response to chemotherapy links with TIL counts. Previous studies have highlighted increased infiltrate of TILs in OAC tumours after neoadjuvant chemotherapy when compared to surgery alone (325). We did not find any statistically significant difference between TIL levels in those patients who received neoadjuvant chemotherapy and those that proceeded directly to surgery as a whole. It would be preferable to evaluate TILs in preoperative biopsies to assess response prior to neoadjuvant therapy. This was attempted but was unsuccessful due to the paucity of tumour material in the small diagnostic endoscopic biopsies unlike the diagnostic biopsies

taken for other tumours that tend to be larger for example breast and colon cancer. However different and heterogeneous chemotherapy regimens were used in previous studies making comparison difficult. We did find that patients who had a significant response to neoadjuvant chemotherapy (as assessed by TRG and LN response) had a higher level of immune infiltrate. This important finding has been shown in other cancer sites where loss of CD4+ Treg [23] and TIL numbers at the margins of liver metastases [24] predicted for response to chemotherapy (523-525). The potential immunological mechanisms by which cytotoxic chemotherapy can provide anti-tumour activity are being increasingly highlighted. These include: subverting immunosuppressive mechanisms; exerting stimulatory effects in immune cells and modulating dying tumour cells so they regain visibility to the host immune response (526). The combination of immunological therapies with conventional chemotherapy has been suggested to provide a synergistic effect if the host immunological response is harnessed appropriately (527). In addition to the effects of chemotherapy on TILs, previous studies have highlighted spontaneous pathological regression of tumours in 13.7% (n=17/124) of OAC cases that have received no neoadjuvant chemotherapy when using TRG to assess the resected tumour specimen. This may represent the host immunological response and successful immune attack (91). Our study would support this hypothesis as significant response to NAC was associated with increased TILs most notably with CD8+ TILs. In other tumour sites CD8+ infiltration has been shown to correlate with specific immunogenic antigen expression and improved survival (528). These findings highlight the T cell effector potential and a means to harness the patient's immune response in cancer.

It is also of interest that increased frequencies of TILs correlates to improved response following the use of Trastuzumab (529) in light of the ToGA study (530) that may lead to the adjuvant use of Trastuzumab in OAC. This adjuvant therapy may lead to a further increase in the adaptive immune response in the tumour as seen in breast cancer (531). We have previously shown that patients are more likely to respond to chemotherapy if patients nutritional blood borne levels are normal (serum albumin) by assessing systemic markers of nutrition and the inflammatory response (Neutrophil/Lymphocyte ratio, serum albumin) (532). It is suggested that suboptimal immunological and nutritional status may contribute to tumour development through subversion of tumour immunity (480, 485) and this is particularly pertinent to OAC. We found that patients with lower TIL levels were less likely to respond to chemotherapy and had lower serum albumin levels. From our data it is not possible to know whether high TIL levels leads to a greater responsiveness to chemotherapy or chemotherapy leads to a higher number of TILs in those that respond and it may well be a combination of the two. The association of poor nutritional status with lower TIL levels and a lack of response is logical and may well be the most likely explanation.

Limitations of this study include its retrospective nature and the associated biases on selection and collection. Patients were excluded if they had had a complete pathological response to neoadjuvant chemotherapy due to the lack of tumour in the resection specimen and also if there was insufficient material collected. However, this cohort is representative of current clinical practice. We found increasing nodal burden (p<0.0001) to be the best independent prognosticator for worse survival. The cohort was homogenous in terms of staging, histology and treatment alogorithms. In addition, patient, tumour factors and survival data are in keeping with published western cohorts making our findings applicable to these populations. The excluded patients with complete pathological response (TRG1) potentially will have had the highest immune response with high TIL levels. This hypothesis is supported by analysis of the cytotoxic response of patients with a complete pathological response in breast cancer patients. Granzyme B and TiA1, cytolytic granules, expressing cells were observed at higher frequency in specimens that had undergone a pathological complete response (523).

Additional criticism could be expressed with regard to the use of TMA cores not being representative of the tumour as a whole. However, multiple studies have utilized this approach and excellent correlation between the two has been established (533). With respect to scoring TILs the technique used has been described frequently by other studies with good inter-observer and intra-observer correlation. Furthermore the data was analysed with and without dichotimization so as to limit false positive results (491, 492).
An unexpected finding in our study was the association of higher TILs for some subsets (CD4+, CD8+ and FOXP3+) in female patients. This may represent a type 1 error, however differences in immune cell numbers with age and sex is well documented. Possible explanations include the inhibitory effect of oestrogen on T-suppressor cells or its stimulatory effect on T-helper cells (534). However this speculation is outwith the remit of this study and will require further analysis.

With regard to our novel finding it remains to be seen whether the composition of TILs can predict for response to chemotherapy prior to treatment and the functional role the immune response plays in improving the response to chemotherapy. Emerging evidence form other solid organ tumours suggests that this might be possible (535). However, as yet the required sensitivity and specificity have not been met for this to enter clinical practice. A potential clinical application of the TIL response could be in the selection of patients for specific adjuvant therapies, specifically using immunomodulators. In our series, a significant response to chemotherapy (TRG2 or LN downstaging) was associated with high TIL levels, suggesting these patients may benefit from further adjuvant chemotherapy. It would be intriguing in this group in particular to evaluate the long term outcome after the use of immunomodulators, that release pre-existing anti-tumour T cells, as has been proposed for aPD1 and aCTLA4 therapies. In contrast, the group of patients who had no or a partial response to chemotherapy (TRG3-5 or no LN

downstaging) with high TIL levels in the pathological specimen additionally may benefit from further adjuvant or alternative chemotherapy in combination with an immunomodulator. In contrast those patients with low TILs may warrant the use of 2nd Line therapies but it seems less likely that immune attack will be successful, unless 2nd line therapy can overcome the barriers to immunological visibility of the cancer.

In summary, the results of this study show that local immune responses, in particular the adaptive immune response, are important independent predictors of cancer specific survival and disease free survival in patients with OAC undergoing radical treatment. These findings highlight the role of the adaptive immune response in preventing tumour recurrence in OAC. A generalized immune response was observed with high correlation between TIL subtypes and at all stages of disease. This is pertinent with regard to the design of therapies as it suggests all patients would benefit from treatment that appropriately boosts the immune response. A significant response to neoadjuvant chemotherapy was associated with higher infiltration with TILs post therapy. Further work analyzing the function of these TIL subsets and the correlation of pretreatment TIL densities with response to therapy may unearth predictors of response to chemotherapy in addition to aid design of novel treatments or as a mechanism to improve response to current therapies.

9. Chapter 9: Cancer testis antigens as potential biomarkers and targets for vaccination in oesophageal adenocarcinoma

9.1. Introduction

The majority of patients in the UK with oesophageal cancer, in the past, were diagnosed with SCC of the thoracic oesophagus. Now the most common type is AC of the lower oesophagus and GOJ with age-standardised incidence rates of AC rising by just under 40% every 5 years (21, 22). The UK has the highest incidence of OAC globally, at 7 cases per 100,000 of the population (22, 23). Moreover, cancer of the oesophagus and GOJ is now the 6th most common cause of cancer death and is associated with a 5-year survival of less than 15% (2). This poor prognosis is due to the majority of patients presenting late with metastatic disease or being medically unfit, with only one in five patients deemed suitable for radical and potentially curative treatment. There is a precursor lesion, Barrett's oesophagus, which is associated with a 30−125 fold greater risk of developing adenocarcinoma (106-108).

For fit patients presenting with operable oesophageal cancer the current standard of care in the UK is neoadjuvant chemotherapy, NAC, followed by resection. Oesophagectomy is performed as the primary curative treatment for local and loco-regional oesophageal cancer (82). Morbidity and mortality occur in significant numbers following oesophageal resection and neoadjuvant chemotherapy despite improvements in preoperative staging (150), patient selection (151), surgery (152) and perioperative care(153). Major complications, including AL, are reported at rates of 3.8-15% (154, 155) with mortality rates varying between 0% and 11% in modern series (156, 157).

To significantly improve on the overall 7-15% 5 year survival of patients presenting with oesophageal cancer, a multi-faceted approach is required. Such strategies will include prevention, identification and surveillance of high risk groups, early diagnosis and improved treatments.

In terms of novel treatments for oesophageal cancer, immunotherapy is attractive, especially for cancers with poor prognosis from current treatment modalities. There is compelling data from recent studies in human cancers that the presence of an immune response predicts improved survival. However it is also clear that many tumours develop strategies to avoid immunosurveillance and acquire the ability to evade or suppress the immune response and current immunotherapies have not delivered significant results in solid tumours.

Cancer testis antigens (CTAs) are particularly attractive as they have potential to: be a biomarker of disease progression; identify high risk groups for surveillance; act as a prognostic biomarker and be a target for immunotherapy. This is due to their restriction of expression and known immunogenicity in patients with cancer. Although a wide range of CTAs have been described, knowledge of their differential expression in OAC is limited. A number of studies have been performed to identify CTAs in oesophageal cancer (250). However these have focused on Asian populations and been restricted to SCC and are

not representative of the population seen in the UK. It is therefore unknown whether immunotherapy may be a suitable therapeutic modality for AC tumours in a UK population. The studied CTAs in oesophageal cancer and gastric AC are summarised in chapter 1 (Table 1-5, Chapter 1).

CTAs may be particularly suited to the role of biomarkers of progression as it has been demonstrated that their expression is associated with increasing stage of disease in solid cancers. This is believed to be due to random promoter demethylation which is associated with disease progression (536). Furthermore, the finding that CTAs are expressed in mesenchymal and human embryonic stem cells suggests that they have a role in cancer cell development and cell differentiation (537-539), hence their potential role as targets for immunotherapy and biomarkers of disease progression and tumourgenesis.

In order to develop cancer immunotherapies, using TAA to boost the immune response, antigen specific epitopes that are recognised by HLA molecules are required to present peptides to the TCR and produce a CTL response. The gene frequency of HLA*A02 is high amongst a variety of ethnic groups and so epitopes that are recognised by this specific HLA molecule are often chosen to identify epitopes suitable for immunotherapy (540).

To test these hypotheses we aimed to perform the first wide screening for CTA expression in all stages of oesophageal AC from BO through to OAC and to explore HLA*A02 specific epitopes from CTAs identified to be overexpressed in OAC and BO.

9.2. Method

9.2.1. Patient samples

Fresh and formalin fixed paraffin embedded tissue representing normal oesophagus, BO and OAC were prospectively collected from patients with normal oesophagus, BO and OAC presenting to UHSFT at presentation endoscopy and at time of surgical resection. PBMCs and serum were also collected at these time points and at the patient's first surgical follow-up clinic appointment. The approval of local ethics committee was obtained (REC No. 09/H0504/66) (Chapter 2.2.3.).

Representative human oesophageal cell lines (OE19, OE21, OE33, HET1A and FLO-1) were obtained from Mr TJ Underwood (University of Southampton) (Chapter 2.2.2.).

For analysis of CTA expression a prospectively collected database of consecutive patients undergoing oesophagogastric resection for AC treated at UHSFT between January 2005 and December 2010 was reviewed. All patients followed a contemporary treatment pathway with description of general parameters included and analysed included in chapter 2 (Chapter 2, 2.2.3).

9.2.2. RNA isolation and cDNA preparation

Total RNA was extracted from cell line pellets or tissue, after disruption and homogenisation (Chapter 2, 2.3.3.1.), with a RNeasy Mini Kit (Chapter 2, 2.3.3.2.) and 1 µl (1ng-5µg) of total RNA was reverse transcribed to cDNA using oligo-dT (Chapter 2, 2.3.3.3.). Quality and quantification of mRNA and cDNA was performed using a Nanodrop ND-1000 spectrophotometer (Chapter 2, 2.3.3.4.).

9.2.3. Reverse transcription polymerase chain reaction

Primer design for all CTAs examined except CTAGE1, an intronless gene, spanned intronic DNA (Chapter 2, 2.3.3.5.). Expression of CTA gene expression was examined by nested RT-PCR for 30 cycles in each round of amplification with β-actin primers and human testis cDNA used as controls. 1 µl of cDNA was used in the first round of amplification with 5 µl of the resulting PCR product used in the second round of amplification (Chapter 2, 2.3.3.6.1.). The final RT-PCR product was then visualised on ethidium bromide stained agarose gels (Chapter 2, 2.3.3.7.).

To examine intraclonal CTA gene expression, single cells of two cell lines (OE21 and OE33) were obtained by flow cytometry deposition (Chapter 2, 2.3.4.1.) and were analysed by nested RT-PCR (Chapter 2, 2.3.4.3.).

For one CTA, PASD1, nested RT-PCR was performed to differentiate which splice variants were expressed using specific primers (Chapter 2, 2.3.3.5).

Experiments were run in duplicate or triplicate and scored as follows. Where two reactions were performed, samples were only counted as positive if they gave a positive result in both reactions and the negative controls were consistently negative. Where three reactions were performed, those specimens for which two out of three reactions gave a positive result but all the negative controls were negative, were counted as positive samples. Water was routinely included as negative controls while mRNA of Testis was used as a positive control. For the one intronless gene, CTAGE1, a no RT control was used to ensure the positive results were not due to expression from genomic DNA.

Amplification products from bulk populations or single tumour cells were eluted and identity confirmed by DNA sequence analysis for at least 5 positive RT-PCR products when available (Chapter 2, 2.3.5).

9.2.4. Quantitative polymerase chain reaction

qRT-PCR used Taqman probe assays (Chapter 2, Table 2-11). Relative expression was calculated against a reference sample of a normal oesophageal biopsy from a healthy patient, as $2^{\Delta\Delta CT} = 2^{(\text{Ct of gene of interest} - \text{Ct of reference gene})}_{\text{control sample}} - (\text{Ct of gene of interest} - \text{Ct of reference gene})_{\text{test sample}}$.

9.2.5. Immunohistochemistry

4 µm sections of TMA blocks were used to assess CTA protein expression. The ABC method of immunoperoxidase labelling was used to identify expression of antigens using either a manual or automated protocol (Chapter 2, 2.3.6). Within each core only tumour cells or representative areas of normal oesophagus or BO were scored. The incorporation of 3 cores on the TMAs for each patient and area of interest ensured representative areas were available for scoring (Chapter 2, 2.3.6.10.).

All sections were digitally image captured to enable sections to be scored under the supervision of experienced pathologists. Scoring of CTAs was done in four levels: negative 0%; low <25%; moderate ≥25-50% and high ≥50%. The intensity of staining was scored if it varied between sections using a similar four point scale: 0 for no staining; 1 = mild; 2 = moderate and 3 = strong.

Following scoring, all expression data needed grouping to facilitate statistical analysis. This was done using justifiable cut-offs (high-low/positive-negative/either side of the median) or quartiles (Chapter 2, 2.3.6.9.1.).

9.2.6. Immunological Antibody assay

A novel immunological assay developed by Serametrix Corporation was used to detect the presence of antibodies to a panel of tumour associated antigens including cancer testis antigens.

9.2.7. Human cytotoxic T lymphocyte responses against CTAs

Tumour antigen-specific T cell responses were assessed by ELISPOT and tetramer assays with (10 days) and without in vitro stimulation of select CTA peptides in medium containing recombinant IL-2 (Chapter 2, 2.3.7).

9.2.8. Statistical analysis

Statistical analysis was performed with SPSS, version 19.0. To test for differences between independent groups the Pearsons chi-squared test, Mann-Whitney and Kruskal-Wallis tests were used with the Wilcoxon signed-rank test used to test between related groups. DFS and OS was analysed using the Kaplan-Meier method and differences between group's survivals were evaluated with the 2 sided log-rank statistic. A multivariate cox regression model was performed to identify the prognostic impact of CTA expression with survival. Covariates found to have prognostic influence on survival with univariate analysis were included to make the final model. *P*-values of <0.05 were considered statistically significant for all analyses (Chapter 2, 2.3.9).

9.3. Results

9.3.1. CTA gene expression in oesophageal cell lines

RT-PCR analysis of three oesophageal cancer cell lines (OE19, OE21 and OE33), one normal immortalised oesophageal cell line (HET1A) and one primary cell line established from a normal oesophageal biopsy showed CTAs to be broadly expressed, on the mRNA level, in all cancer cell lines with no expression in the cells grown from the normal oesophageal biopsy (Table 8-1). All classes of CTAs were expressed in the oesophageal cancer cell lines.

Table 9-1: Expression of CTA genes in oesophageal cell lines and isolates by RT-PCR

	Testis Restricted			Testis/Brain restricted			Testis Selective						
Cell Lines Cell Isolates	**MAGEA2**	**NYESO**	**SAGE1**	**GAGE**	**HORMAD1**	**PASD1**	**BAGE**	**HAGE**	**FATE1**	**LAGE**	**MAGEA3**	**SPAG9**	**No. CTAs Expressed**
OE19	+	+	+	-	+	-	-	-	+	+	+	+	8
OE21	-	-	+	-	-	+	-	-		-	-	-	2
OE33	-	-	-	-	+	+	-	+	+	-	-	-	4
HET1A	-	-	+	-	+	+	-	-	-	-	-	-	2
NOK0210	-	-	-	-	-	-	-	-	-	-	-	-	0

9.3.2. CTA gene expression in normal oesophagus, BO and OAC patients

CTA transcripts were expressed in 5 of 26 (19%) normal oesophageal biopsies, 26 of 27 BO biopsies (96%) and 20 of 20 (100%) in AC of OAC patients at the time of resection. Detailed CTAs transcript co-expression is presented in tables 8-2 to 8-6. CTA transcripts were expressed with low frequency in normal oesophageal tissue from patients with AC and healthy patients with non-specific upper abdominal symptoms warranting endoscopy biopsies (HORMAD1 8%, FATE1 8%, HAGE 4% and LAGE 4%). No testis restricted CTAs were identified in normal tissue with one CTA classed as testis/brain restricted (HORMAD1 8%).

CTA genes were expressed in BO, most frequently PASD1 56%, SPAG9 48%, HORMAD1 37% and FATE1 22%. Representative agarose gel image of PCR amplification of CTA gene transcripts are shown in Figure 9-1. There was a significant increase in expression of CTAs in normal tissue median 0 (range 0-2) to BO median 1 (0-5) (p=0.038) with further increased expression from BO to OAC median 3 (2-7) CTA genes expressed per patient sample (Figure 8-1). Six CTAs were not found to be expressed in any oesophageal tissue (MAGEC2, NY-ESO-1, SSX2, GAGE, MAGEA3 and PAGE3).

The expression profile of particular CTAs in normal oesophageal tissue, BO and OAC is presented in figure 8-3. All genes with expression in BO also were also expressed in OAC with a trend towards increasing CTAs in higher stage of disease (Figure 8-4).

Table 9-2: Expression of CTA genes in normal oesophagus from healthy patients by RT-PCR

	Testis Restricted								Testis/Brain Restricted			Testis Selective							
Patient Sample	CSAG2	MAGEA2	MAGEC1	NYESO	SAGE1	SSX2	CTAGE1	GAGE	HORMAD1	MAGEC2	PAGE3	PASD1	BAGE	FATE1	HAGE	LAGE	MAGEA3	SPAG9	No. CTAs Expressed
No. of +ve samples	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	
O2	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1
O3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
O5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
O6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
O7	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1
O8	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	1

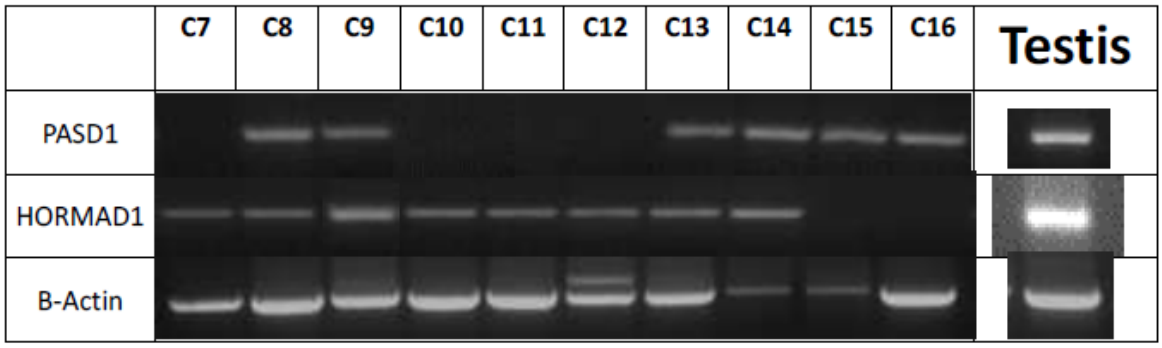


Figure 9-1: Representative agarose gel image of PCR amplification of CTA gene transcripts in oesophageal OAC patients

Table 9-3: Expression of CTA genes in normal oesophagus from OAC patients by RT-PCR

	Testis Restricted CTAs						Testis/Brain Restricted						Testis Selective							
Patient Sample	CSAG2	MAGEA2	MAGEC1	NYESO	SAGE1	SSX2	CTAGE1	GAGE	HORMAD1	MAGEC2	PAGE3	PASD1	BAGE	FATE1	HAGE	LAGE	MAGEA3	SPAG9	No. CTAs Expressed
No. of +ve Samples	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	
NC1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	1
NC6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC7	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	2
NC10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
NC23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0

Table 9-4: Expression of CTA genes in BO from OAC patients by RT-PCR

	Testis Restricted CTAs						Testis/Brain Restricted						Testis Selective							
Patient Sample	CSAG2	MAGEA2	MAGEC1	NYESO	SAGE1	SSX2	CTAGE1	GAGE	HORMAD1	MAGEC2	PAGE3	PASD1	BAGE	FATE1	HAGE	LAGE	MAGEA3	SPAG9	No. CTAs Expressed
No. of Positive Samples	0	0	0	0	0	0	1	0	2	0	0	3	0	1	1	0	0	2	
BC2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
BC5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	1
BC7	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	4
BC13	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
BC14	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	2
BC21	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
BC23	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1

Table 9-5: Expression of CTA genes in BO from BO patients by RT-PCR

	Testis Restricted CTAs								Testis/Brain Restricted			Testis Selective							
Patient Sample	**CSAG2**	**MAGEA2**	**MAGEC1**	**NYESO**	**SAGE1**	**SSX2**	**CTAGE1**	**GAGE**	**HORMAD1**	**MAGEC2**	**PAGE3**	**PASD1**	**BAGE**	**FATE1**	**HAGE**	**LAGE**	**MAGEA3**	**SPAG9**	**No. CTAs Expressed**
No. of +ve Samples	0	0	0	0	0	0	0	0	8	0	0	12	0	5	1	0	0	11	
B2	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
B3	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	2
B4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
B5	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
B6	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	+	5
B7	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
B8	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1
B9	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	+	4
B10	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
B11	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	2
B12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
B13	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	2
B14	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	3
B15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
B16	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1
B17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
B18	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	1
B19	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	3
B20	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	2
B21	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	+	4

Table 9-6: Expression of CTA genes in AC from OAC patients by RT-PCR

	Testis Restricted CTAs						Testis/Brain Restricted					Testis Selective							
Patient Sample	**CSAG2**	**MAGEA2**	**MAGEC1**	**NYESO**	**SAGE1**	**SSX2**	**CTAGE1**	**GAGE**	**HORMAD1**	**MAGEC2**	**PAGE3**	**PASD1**	**BAGE**	**FATE1**	**HAGE**	**LAGE**	**MAGEA3**	**SPAG9**	**No. CTAs Expressed**
No. of +ve Samples	4	2	1	0	1	0	16	0	15	0	0	16	2	1	4	2	1	8	
C1	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	2
C2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	3
C3	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	3
C5	-	-	-	-	+	-	-	-	+	-	-	+	+	-	-	+	-	-	5
C6	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	3
C7	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	4
C8	+	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	5
C9	-	+	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	7
C10	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	4
C11	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	4
C12	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	2
C13	+	-	-	-	-	-	+	-	+	-	-	+	-	+	-	-	-	+	6
C14	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	3
C15	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	3
C16	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	2
C17	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	3
C18	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	3
C19	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	3
C20	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	2
C21	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	2
C22	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	2
C23	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	2

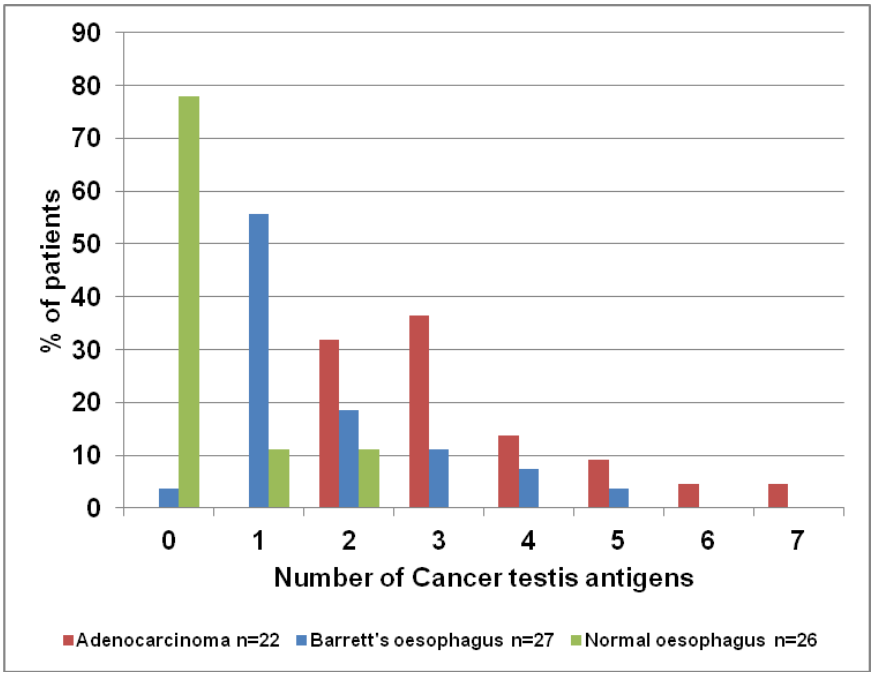


Figure 9-2: Number of CTA genes expressed by tissue type by RT-PCR

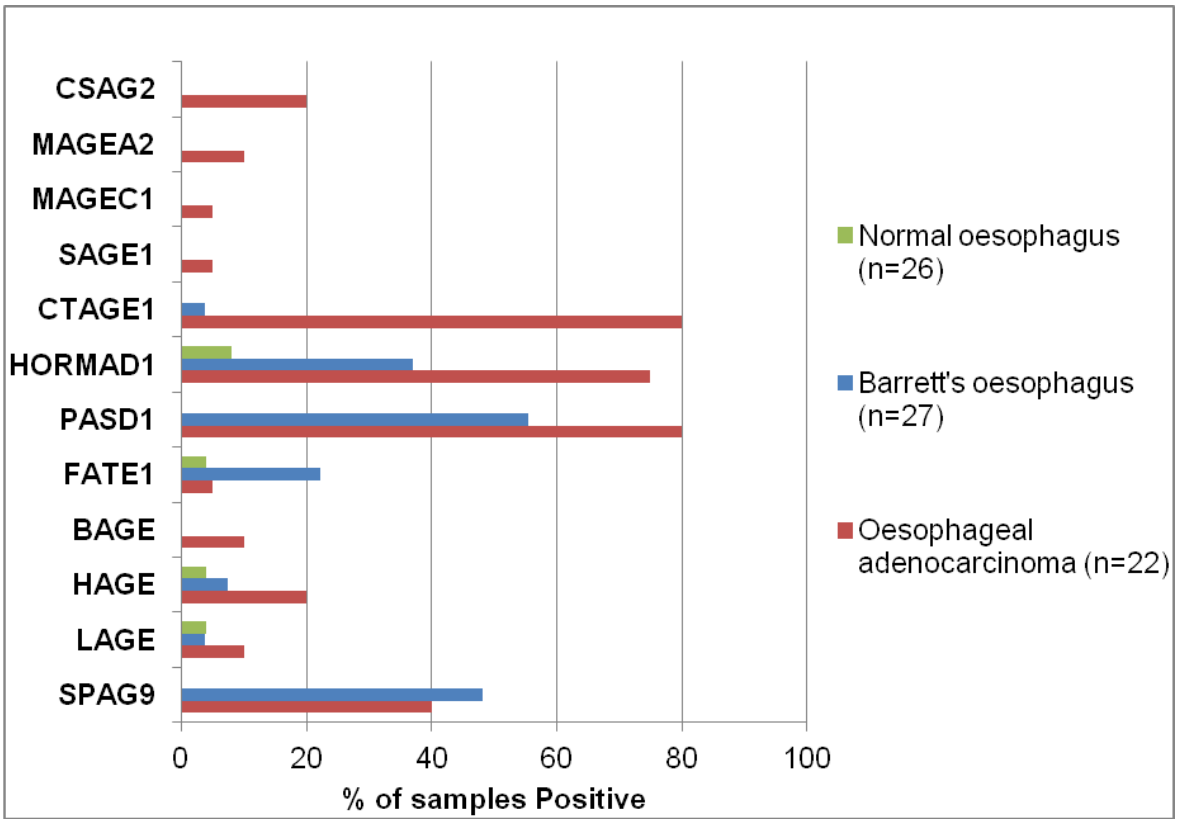


Figure 9-3: Frequency of CTA genes expressed by tissue type by RT-PCR

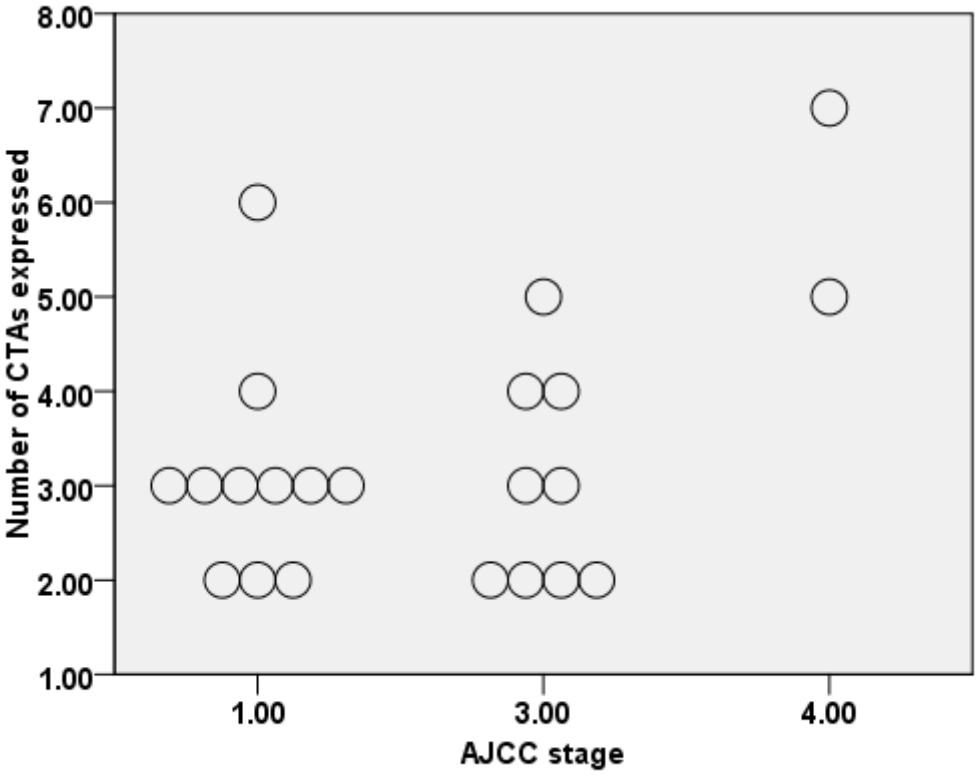


Figure 9-4: Number CTAs expressed according to pathological AJCC stage by RT-PCR

9.3.3. Single cell, splice variant expression and specificity of PASD1 gene transcript expression

Single cell expression of PASD1 transcipts was observed in between 21 and 30 % of tumour cells from 3 cell lines (Figure 8-5). Both variants of the PASD1 gene were expressed in all tissue analysed including 3 cell lines, 5 BO and 5 OAC patients.

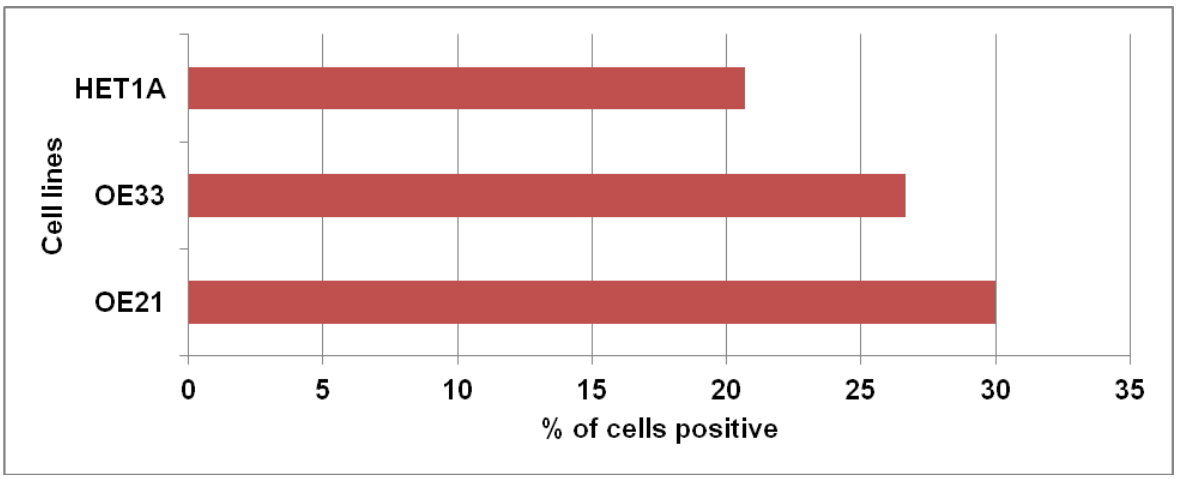


Figure 9-5: Single cell expression of PASD1 gene in oesophageal cell lines by RT-PCR

CTA genes close to and flanking PASD1 on chromosome Xq28 were evaluated for gene coexpression in BO and OAC patients and the results are presented in table 8-7. The closest CTA FATE1 had relatively low co-expression with PASD1 occurring in 6% of OAC (n=1/16) and 20% of BO (n=3/15) patients (Table 8-4 to 8-7).

	Testis/Brain Restricted		Testis Selective			
Patient Sample	**MAGEA8**	**PASD1**	**FATE1**	**MAGEA4**	**MAGEA9**	**No. CTAs Expressed**
No. of Positive Samples	8	10	2	10	10	
Barrett's Oesophagus						
B2	-	+	-	+	+	2
B3	+	+	-	+	+	3
B6	+	+	+	+	+	4
B7	+	+	-	+	+	3
B9	-	+	+	+	+	3
Oesophageal Adenocarcinoma						
C1	+	+	-	+	+	3
C3	+	+	-	+	+	3
C5	-	+	-	+	+	2
C6	+	+	-	+	+	3
C8	+	+	-	+	+	3

Table 9-7: Expression of CTAs close to PASD1 gene on chromosome X by RT-PCR

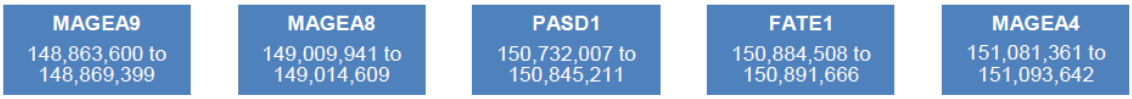


Figure 9-6: Position of flanking CTAs to PASD1 on chromosome X

9.3.4. Sequence analysis of RT-PCR products

Sequence analysis of RT-PCR products confirmed the identity of all CT genes and splice variants investigated.

9.3.5. Quantification of CTA gene expression

The results of positive CTA genes expressed in OAC tissue relative to healthy oesophagus normalised against reference gene GAPDH are presented in figure 9-8. The range of expression of individual CTA genes was from 8% (1/13) to 85% (11/13) from oesophageal AC tissue and showed variability relative to healthy oesophagus normalised against reference gene GAPDH.

The number of tumour antigens expressed in each patient is shown in figure 9-7. All samples expressed tumour antigens with increasing expression in Barrets oesophagus to oesophageal AC.

Comparison of RT-PCR and qRT-PCR in tissue examined for gene expression by both techniques is presented in table 9-8 showing differences in the two techniques.

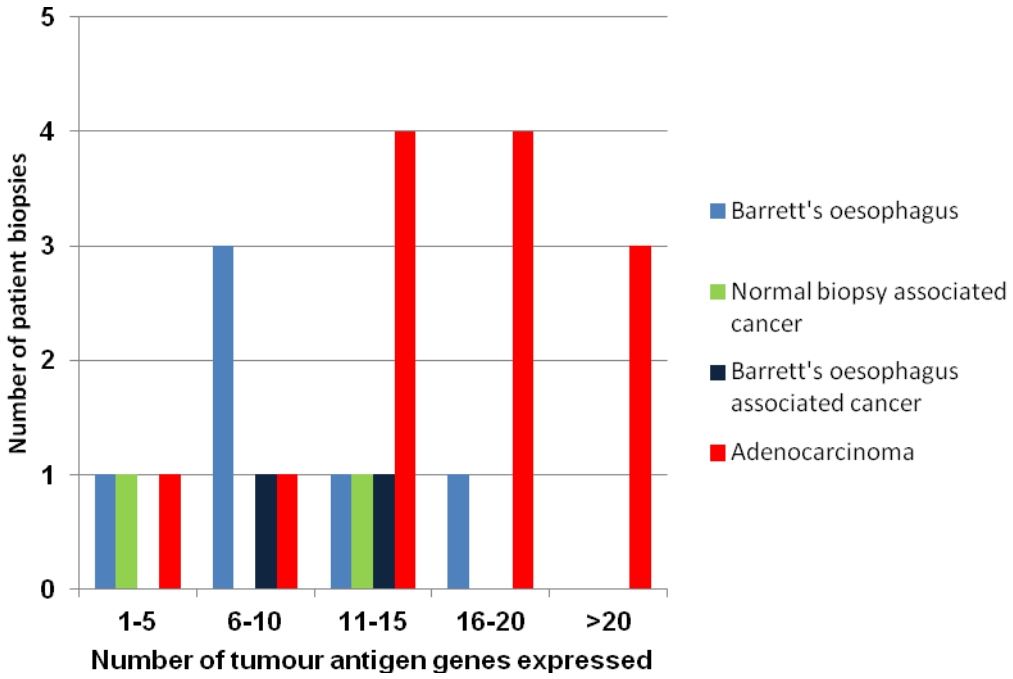


Figure 9-7: Number of Tumour antigen expression in oesophageal primary tissue by qRT-PCR

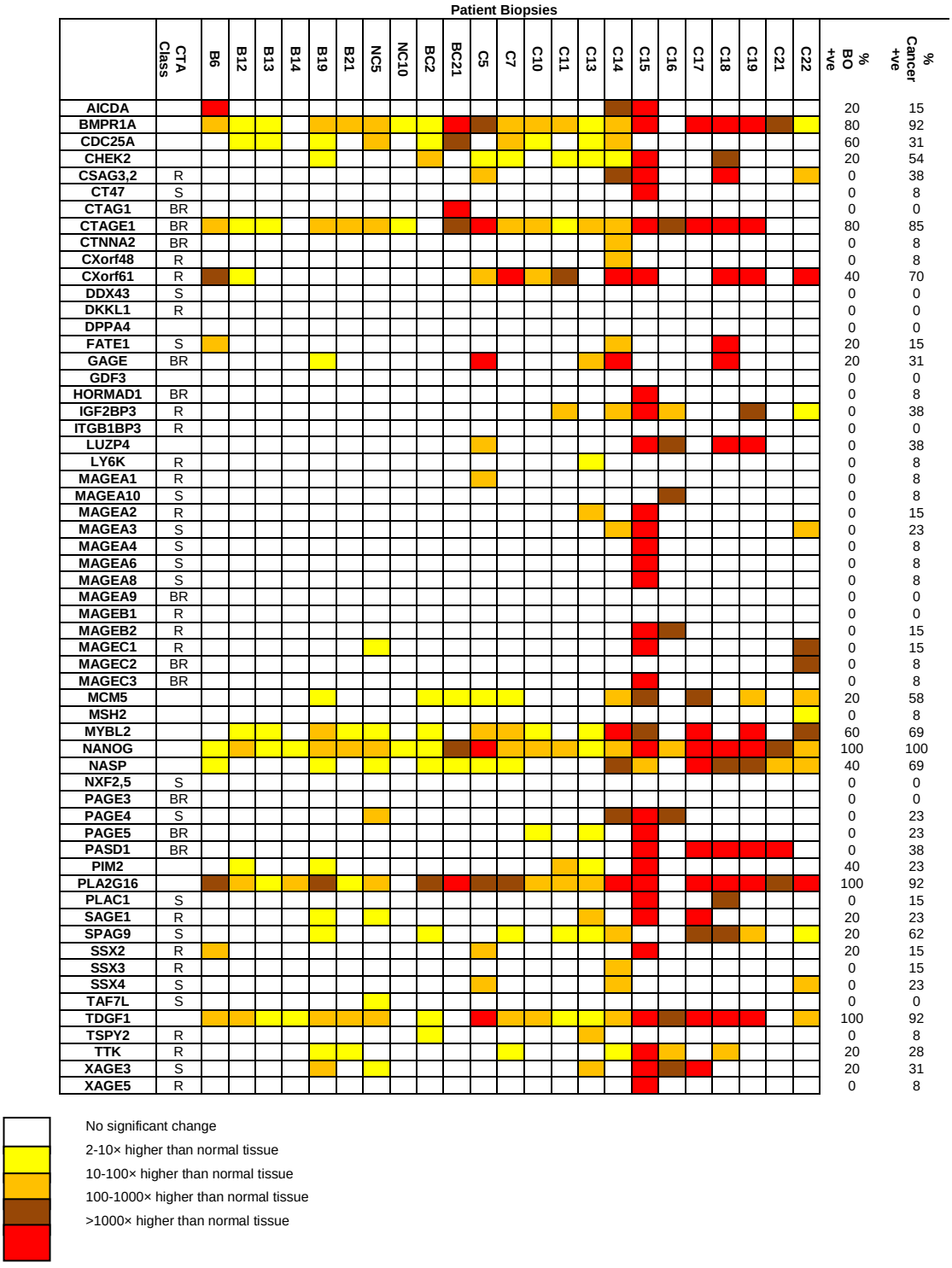


Figure 9-8: Tumour antigen expression in oesophageal primary tissue by qRT-PCR. Gene expression in samples (B – Barrett's Oesophagus; NC – Normal oesophagus associated cancer; BC – Barrett's Oesophagus associated cancer; C- Cancer) is compared to the expression in normal oesophageal tissue from patients referred for endoscopy with normal findings

Table 9-8: Comparison of RT-PCR and qRT-PCR results for cancer testis antigen expression

	Testis Restricted CTAs										Testis/Brain Restricted												Testis Selective					
	MAGEA2		MAGEC1		NYESO		SAGE1		SSX2		CTAGE1		GAGE		HORMAD1		MAGEC2		PAGE3		PASD1		FATE1		MAGEA3		SPAG9	
	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR	RT-PCR	q-PCR
B6	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	+	+	-	-	+	-
B12	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
B13	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
B14	-	-	-	-	-	-	-	-	-	-	-		-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-
B19	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+
B21	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-
NC5	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NC10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BC2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
BC21	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C5	-	-	-	-	-	-	+	-	-	+	-	+	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
C7	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
C10	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
C11	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
C13	-	+	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	+	-	+	-	-	-	+	+
C14	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	+	-	-	+	-	+	-	+
C15	-	+	-	+	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-
C16	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-		-	-	-	-	+	-	-	-	-	-	-	-
C17	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	+
C18	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+	+
C19	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	+
C21	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
C22	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	+

9.3.6. CTA protein expression in normal oesophagus, BO and OAC

Immunohistochemistry was positive for all CTAs with frequency ranging from 0.5% to 11% (NY-ESO1 n=10/198; PASD1 n=16/198; CTAGE1 n=17/154; SPAG9 n=8/156; MAGEC1 n=6/198; MAGEA3 n=11/198; MAGEA9 n=5/198; HORMAD1 n=1/198). NY-ESO1 positive staining showed poorer disease free survival with estimated DFS of 1.989 years (95% CI: 0.000-2.776) compared to 4.088 years (95% CI: 2.407-5.769) for negative staining (Figure 9-9). This was not an independent prognositic marker on multivariate analysis with correlation with increased stage of disease (pT stage, p=0.079) and pN stage (*p*=0.081) showing statistically significant association with vascular invasion (*p*=0.011).

The other CTAs IHC staining patterns did not show any correlation with clinic-pathological features and outcomes.

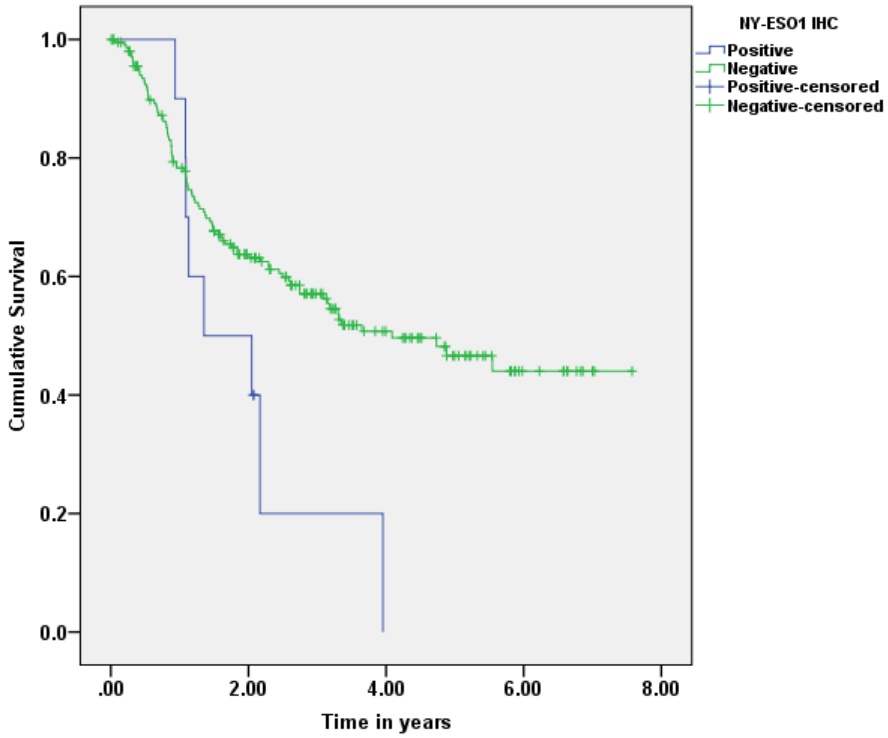


Figure 9-9: Disease free survival according to postoperative NY-ESO1 immunohistochemistry staining (Logrank *p*=0.044)

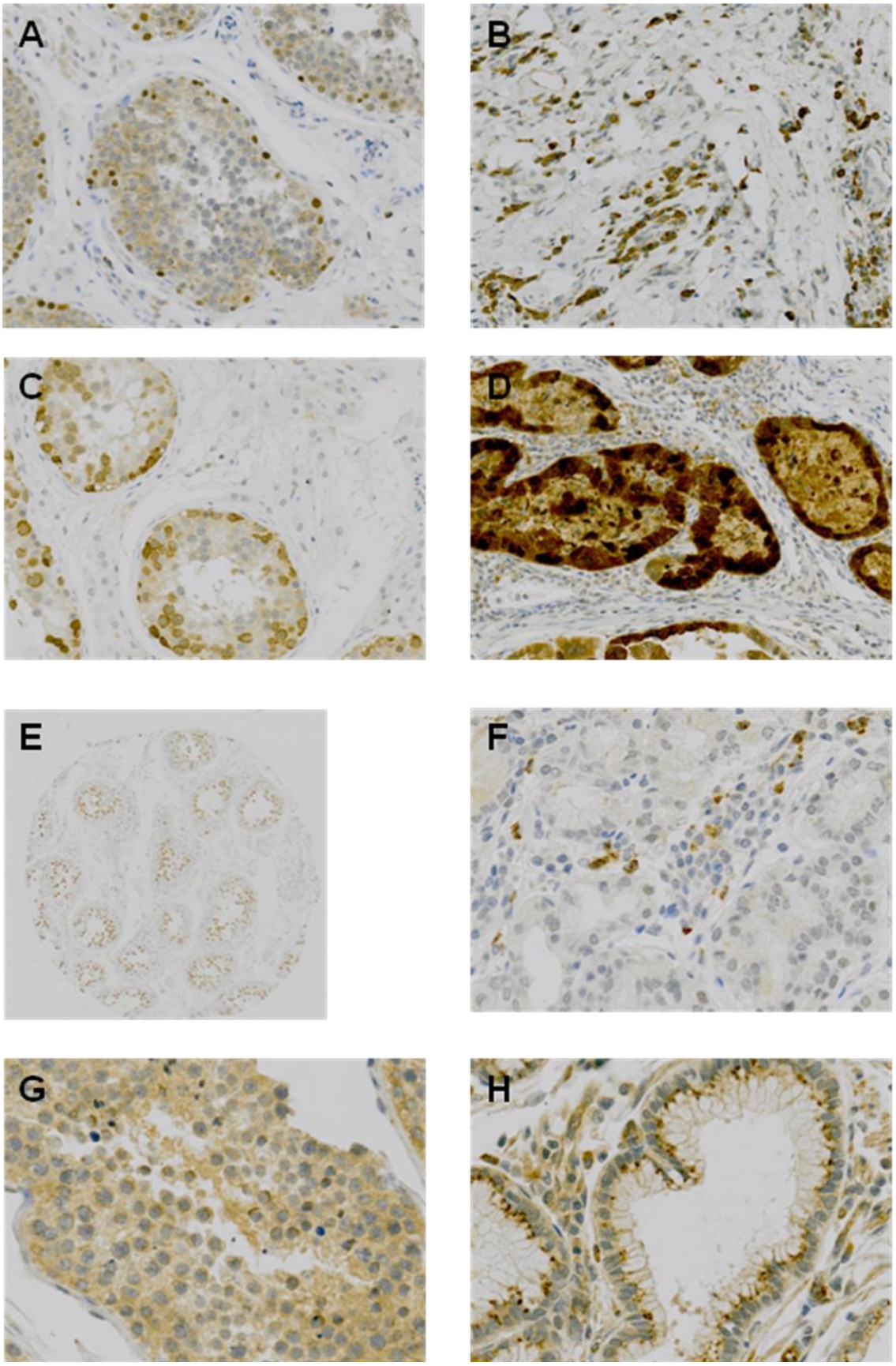


Figure 9-10a: Immunohistochemical staining from tissuemicroarray for PASD1 (A: testis; B: OAC), NY-ESO-1 (C: testis; D: OAC), HORMAD1 (E: testis; F: OAC), SPAG9 (G: testis; H: OAC)

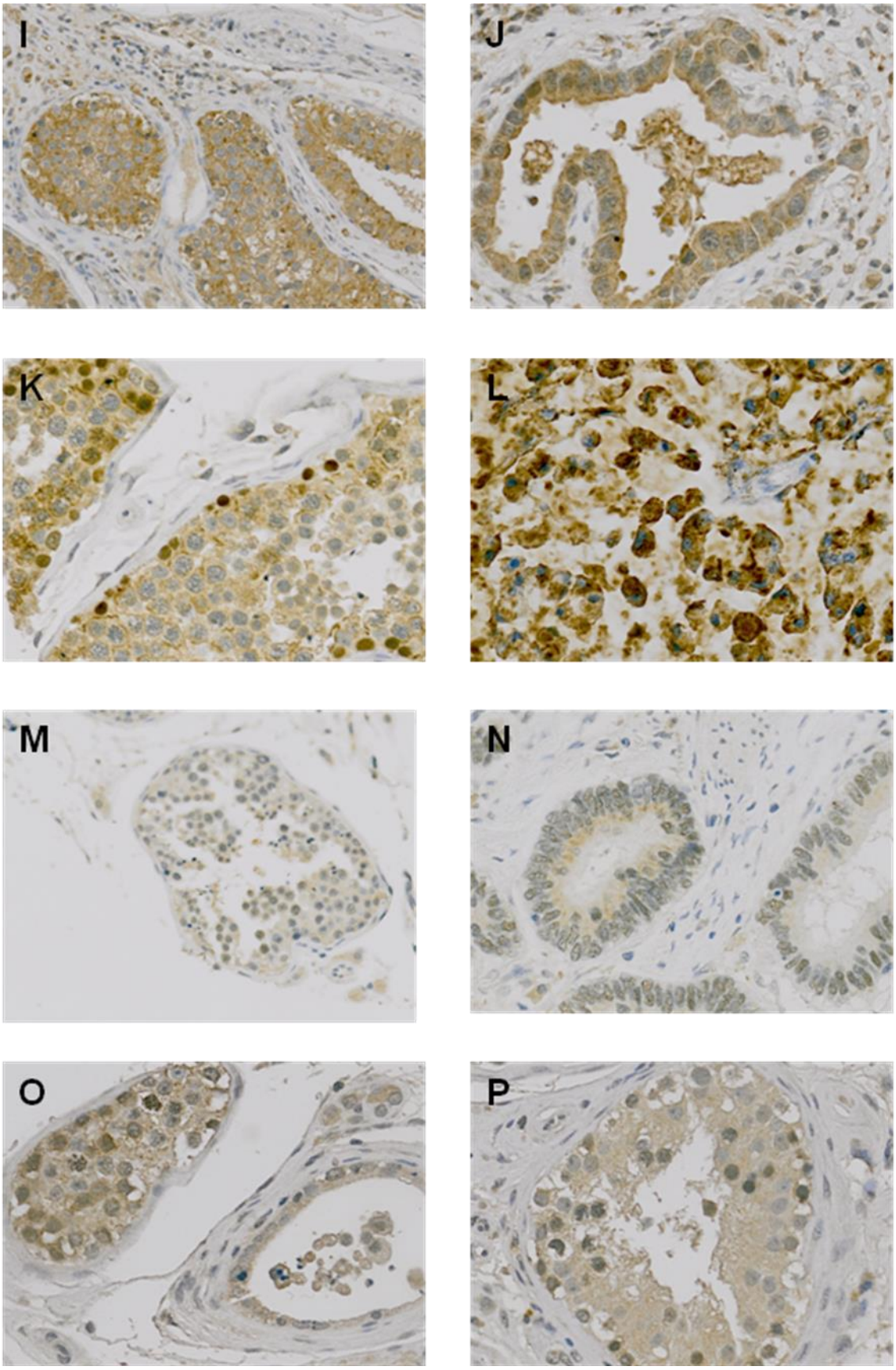


Figure 9-10b: Immunohistochemical staining from tissuemicroarray for MAGEA3 (I: testis; J: OAC), CTAGE1 (K: testis; L: OAC), MAGEC1 (M: testis; N: OAC), MAGEA9 (O: testis; P: OAC)

9.3.7. Humoral tumour antigen specific immunity

The serum of 105 patients were available for analysis (GORD n=7, BO n=9, HGD n=3, AC n=86). The majority of autoantibodies were able to discriminate between some patients with GORD, BO, HGD and AC (Figure 9-11 and Table 9-9). There was considerable coexpression of CTA autoantibodies with greater than 50% of patients with OAC having more than one autoantibody detected in keeping with the gene expression findings. By combining the individual autoantibody responses using a cut-off of 2 s.d. the assay was able to differentiate between GORD and BO or AC with a diagnostic accuracy of 89% and 80% respectively (Figure 9-12 and 9-13).

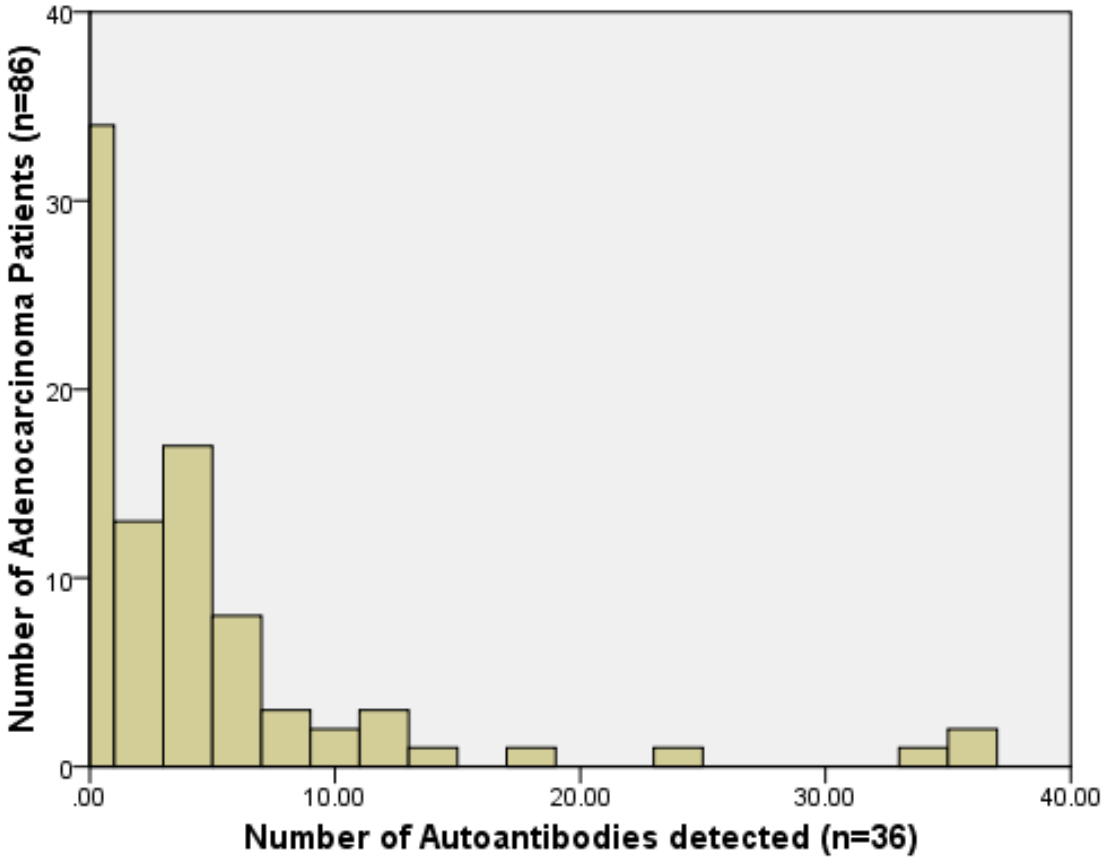


Figure 9-11: Number of tumour autoantibodies detected in individual OAC patients

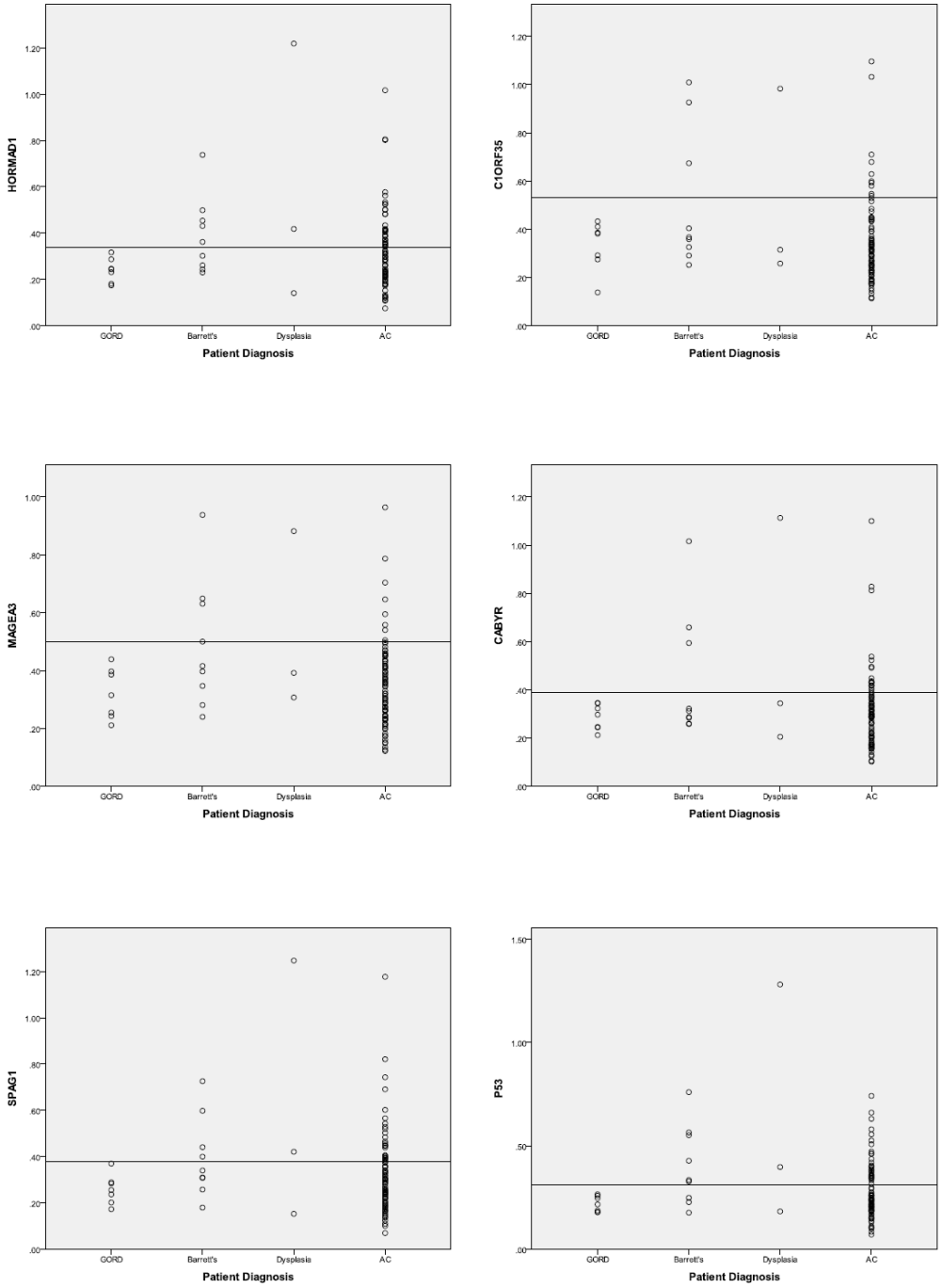


Figure 9-12: Examples of Dot plots of discriminatory autoantibodies. Circles represent individual patient serum autoantibodies absolute values. Transverse line representing a cut-off 2 standard deviation (SD) above the values for autoantibodies in patients with GORD

Table 9-9: Relationship of autoantibody expression by tissue type

	Patient Histological type							
	GORD (n=7)	BO (n=9)	HGD (n=3)	AC (n=86)	GORDvsBOvsHGDvsAC	GORDvsAC	GORDvsBO	BOvsAC
	% > 2 SD above the mean GORD value	% > 2 SD above the mean GORD value	% > 2 SD above the mean GORD value	% > 2 SD above the mean GORD value	*p* value	*p* value	*p* value	*p* value
BIRC5	0	44.4	33.3	15.1	0.073	0.267	0.042	0.029
C1ORF35	0	33.3	33.3	11.6	0.142	0.340	0.090	0.071
CABYR	0	33.3	33.3	19.8	0.379	0.193	0.090	0.342
CSAG2	0	33.3	33.3	4.7	0.050	0.560	0.090	0.002
CTAG2	0	33.3	33.3	8.1	0.046	0.432	0.090	0.019
ctage1 10	0	22.2	0	9.3	0.433	0.399	0.182	0.229
ctage1 20	0	22.2	0	14.0	0.535	0.290	0.182	0.506
ctage1 50	0	22.2	0	12.8	0.526	0.314	0.182	0.433
CTNNB1	0	44.4	33.3	11.6	0.026	0.340	0.042	0.008
GAGE1	0	33.3	33.3	4.7	0.050	0.560	0.090	0.002
GAGE8	0	33.3	33.3	7.0	0.026	0.470	0.090	0.010
HORMAD1	0	55.6	66.7	36.0	0.084	0.052	0.017	0.251
MAGEA1	0	33.3	33.3	3.5	0.001	0.615	0.090	<0.0001
MAGEA3	0	33.3	33.3	9.3	0.073	0.399	0.090	0.032
MAGEA4	0	44.4	33.3	15.1	0.073	0.267	0.042	0.029
MAGEB6	0	44.4	33.3	4.7	<0.0001	0.560	0.042	<0.0001
MAGEC1	0	33.3	33.3	15.1	0.259	0.267	0.090	0.165
NLRP4	0	33.3	33.3	12.8	0.181	0.314	0.090	0.098
NYESO1	0	33.3	33.3	11.6	0.142	0.340	0.090	0.071
P53	0	66.7	66.7	36.0	0.034	0.052	0.006	0.073
PASD1p10	0	11.1	0	3.5	0.630	0.675	0.362	0.279
PASD1p20	0	22.2	0	7.0	0.308	0.470	0.182	0.117
PASD1p50	0	11.1	0	11.6	0.730	0.340	0.362	0.963
PASD1pro10	0	0	0	3.5	0.877	0.615	na	0.569
PASD1pro20	0	11.1	0	5.8	0.778	0.512	0.362	0.534
PASD1pro50	0	22.2	0	4.7	0.149	0.560	0.182	0.039
PRAME	0	44.4	33.3	19.8	0.157	0.193	0.042	0.090
SOX2	0	33.3	33.3	16.3	0.295	0.247	0.090	0.204
SPAG1	0	44.4	66.7	27.9	0.109	0.105	0.042	0.301
SPANXA1	0	33.3	33.3	5.8	0.012	0.512	0.090	0.005
SPANXB1	0	33.3	33.3	7.0	0.026	0.470	0.090	0.010
SSX2	0	33.3	33.3	3.5	0.001	0.615	0.090	<0.0001
SSX4	0	33.3	33.3	4.7	0.005	0.560	0.090	0.002
SSX5	0	33.3	33.3	4.7	0.005	0.560	0.090	0.002
TSSK6	0	33.3	33.3	18.6	0.355	0.210	0.090	0.293
XAGEA2	0	33.3	66.7	4.7	0.005	0.560	0.090	0.002
Any Biomarker	0	77.8	66.7	60.5	0.010	0.002	0.002	0.308

GORD – Gastro oesophageal reflux disease; HGD - High grade dysplasia; AC - adenocarcinoma; BO Barrett's oesophagus; p values using Pearson χ^2, Mann Whitney U test or Kruksal Wallis test as appropriate

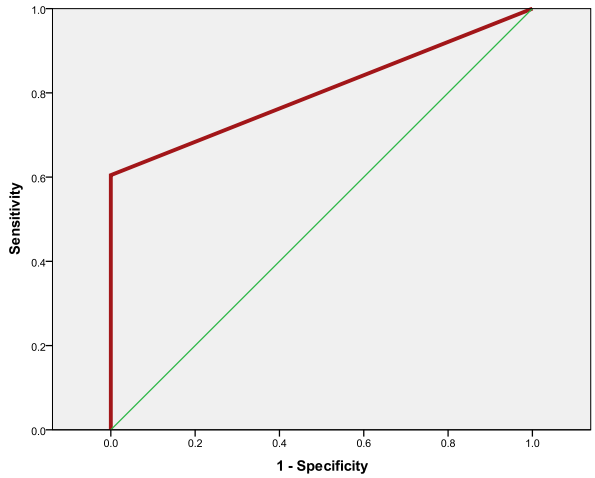


Figure 9-13: Receiver operating curve (ROC) for the number of autoantibody above the cut-off (2 SD) detected with regard to differentiating between GORD and AC with respective area under the curve (AUC) value of 0.802 (95% CI: 0.695-0.910) *p*=0.008. When all the biomarkers are combined a sensitivity of 61 % (52/86) and a specificity of 100% (7/7) is achieved

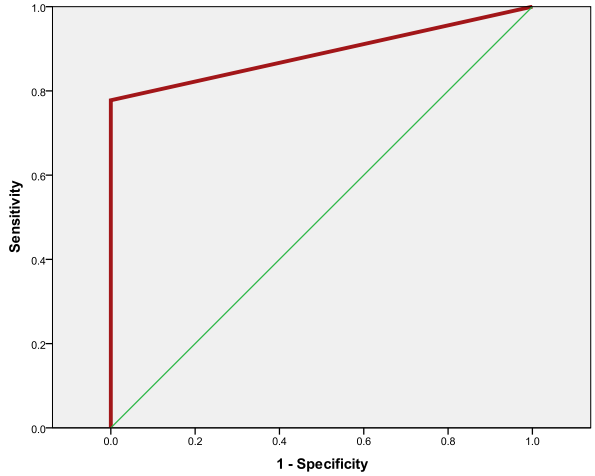


Figure 9-14: Receiver operating curve (ROC) for the number of autoantibody above the cut-off (2 s.d.) detected with regard to differentiating between GORD and BO with respective area under the curve (AUC) value of 0.889 (95% CI: 0.714-1.000) *p*=0.010. When all the biomarkers are combined a sensitivity of 77.8 % (7/9) and a specificity of 100% (7/7) is achieved

9.3.8. Immunogenicity of selected CTAs in oesophageal cancer patients

9.3.8.1. Selection of potential HLA-A*0201 epitopes for selected CTAs

Based on high binding prediction scores by at least two programs peptides were selected for assessing antigen specific immune responses (Table 8-8).

9.3.8.2. Induction of antigen-specific CTL response by CTA derived peptides

We attempted to induce CTLs recognising specific CTA derived peptides in the context of HLA-A*0201, a common allele in the general population. PBMCs, from patients

who displayed or did not display gene expression in their tumour by RT-PCR, were assessed for CD8+ response and IFN-Ɣ production *in-vitro*. This was performed on PBMCs before and after stimulation with each specific peptide.

We were unable to demonstrate antigen-specific CTL response against the PASD1 derived peptide (QLLDGFMITL) in the 10 patient's positive for PASD1 gene transcript in their tumour by virtue of no production of an IFN-Ɣ response *in vitro*. We found a CD4+ and CD8+ response that was not observed in healthy patients.

In patients, positive for CTAGE1 gene transcript in the tumour, CTL responses to CTAGE1 were observed in a small number of patients with CD4+ and CD8+ responses in 3/3 patients and 2/3 patients producing an IFN-Ɣ response *in vitro*. The uncertainty of the specificity of the tetramer assay is highlighted due to the simultaneous CD4+ response making it uncertain as to the true positivity of these findings although this was not observed in healthy patients. The results of antigen-specific responses are presented in detail in table 8-9 with representative analysis in figures 8-8 to 8-11.

Table 9-10: Prediction of HLA-A*0201 restricted peptides from CTAGE1, MAGEA3 and PASD1 by SyFPEITHI, BIMAS and NetCTL

		Scores		
Peptide	**Sequence**	**SyFPEITHI**	**BIMAS**	**NetCTL**
CTAGE1	FLWRSFRSV	24	1009.088	0.30
MAGEA3	KVAELVHFL	25	339.313	0.90
PASD1	QLLDGFMITL	25	2832.351	0.25

Table 9-11: Induction of antigen-specific CTL response by CTA derived peptides assessed by CD8+ response to peptide specific tetramers

					Pre-stimulation			Post-stimulation		
	mRNA Status by RT-PCR			HLA type	CD8+ response			CD8+ response		
Patient Sample	CTAGE1	PASD1	MAGEA3	HLA-A2	PASD1	CTAGE1	MAGEA3	PASD1	CTAGE1	MAGEA3
No. of samples positive/total	16/22	16/22	1/22	12/17	0/10	?/3	0/0	0/5	0/0	0/0
C1	-	+	-	+	-	ND	ND	-	ND	ND
C2	-	-	-	+	ND	ND	ND	ND	ND	ND
C3	-	+	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C5	-	+	-	-	-	n/a	n/a	ND	n/a	n/a
C6	-	+	-	+	-	ND	ND	-	ND	ND
C7	+	-	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C8	+	+	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C9	+	+	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C10	+	-	-	-	n/a	n/a	n/a	n/a	n/a	n/a
C11	+	-	-	+	ND	?	ND	ND	ND	ND
C12	+	-	-	+	ND	?	ND	ND	ND	ND
C13	+	+	-	-	-	n/a	n/a	ND	n/a	n/a
C14	+	+	-	-	-	n/a	n/a	ND	n/a	n/a
C15	+	+	-	-	-	n/a	n/a	ND	n/a	n/a
C16	+	+	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C17	+	+	-	+	-	n/a	n/a	-	n/a	n/a
C18	+	-	-	+	ND	?	ND	ND	ND	ND
C19	+	+	-	-	ND	ND	ND	ND	ND	ND
C20	+	+	-	+	-	n/a	n/a	-	n/a	n/a
C21	+	+	-	+	-	ND	ND	-	ND	ND
C22	+	+	-	+	-	n/a	n/a	ND	n/a	n/a
C23	-	+	-	+	n/a	n/a	n/a	n/a	n/a	n/a
HV-11	ND	ND	ND	+	-	-	-	ND	ND	ND
HV1020	ND	ND	ND	+	-	-	-	ND	ND	ND
HV1021	ND	ND	ND	+	-	-	-	ND	ND	ND

Table 9-12: Induction of antigen-specific CTL response by CTA derived peptides assessed by IFN-Ɣ production by ELISPOT assay

					Pre-stimulation			Post-stimulation		
	mRNA Status by RT-PCR			HLA type	IFN-Ɣ production			IFN-Ɣ production		
Patient Sample	CTAGE1	PASD1	MAGEA3	HLA-A2	PASD1	CTAGE1	MAGEA3	PASD1	CTAGE1	MAGEA3
No. of samples positive/total	16/22	16/22	1/22	12/17	0/10	2/3	0/0	0/10	0/0	0/0
C1	-	+	-	+	-	ND	ND	-	ND	ND
C2	-	-	-	+	ND	ND	ND	ND	ND	ND
C3	-	+	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C5	-	+	-	-	n/a	n/a	n/a	n/a	n/a	n/a
C6	-	+	-	+	-	ND	ND	-	ND	ND
C7	+	-	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C8	+	+	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C9	+	+	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C10	+	-	-	-	n/a	n/a	n/a	n/a	n/a	n/a
C11	+	-	-	+	ND	-	ND	ND	ND	ND
C12	+	-	-	+	ND	+	ND	ND	ND	ND
C13	+	+	-	-	ND	n/a	n/a	ND	n/a	n/a
C14	+	+	-	-	ND	n/a	n/a	ND	n/a	n/a
C15	+	+	-	-	ND	n/a	n/a	ND	n/a	n/a
C16	+	+	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a
C17	+	+	-	+	-	n/a	n/a	-	n/a	n/a
C18	+	-	-	+	ND	+	ND	ND	ND	ND
C19	+	+	-	-	ND	ND	ND	ND	ND	ND
C20	+	+	-	+	-	n/a	n/a	-	n/a	n/a
C21	+	+	-	+	-	ND	ND	-	ND	ND
C22	+	+	-	+	-	n/a	n/a	-	n/a	n/a
C23	-	+	-	+	n/a	n/a	n/a	n/a	n/a	n/a

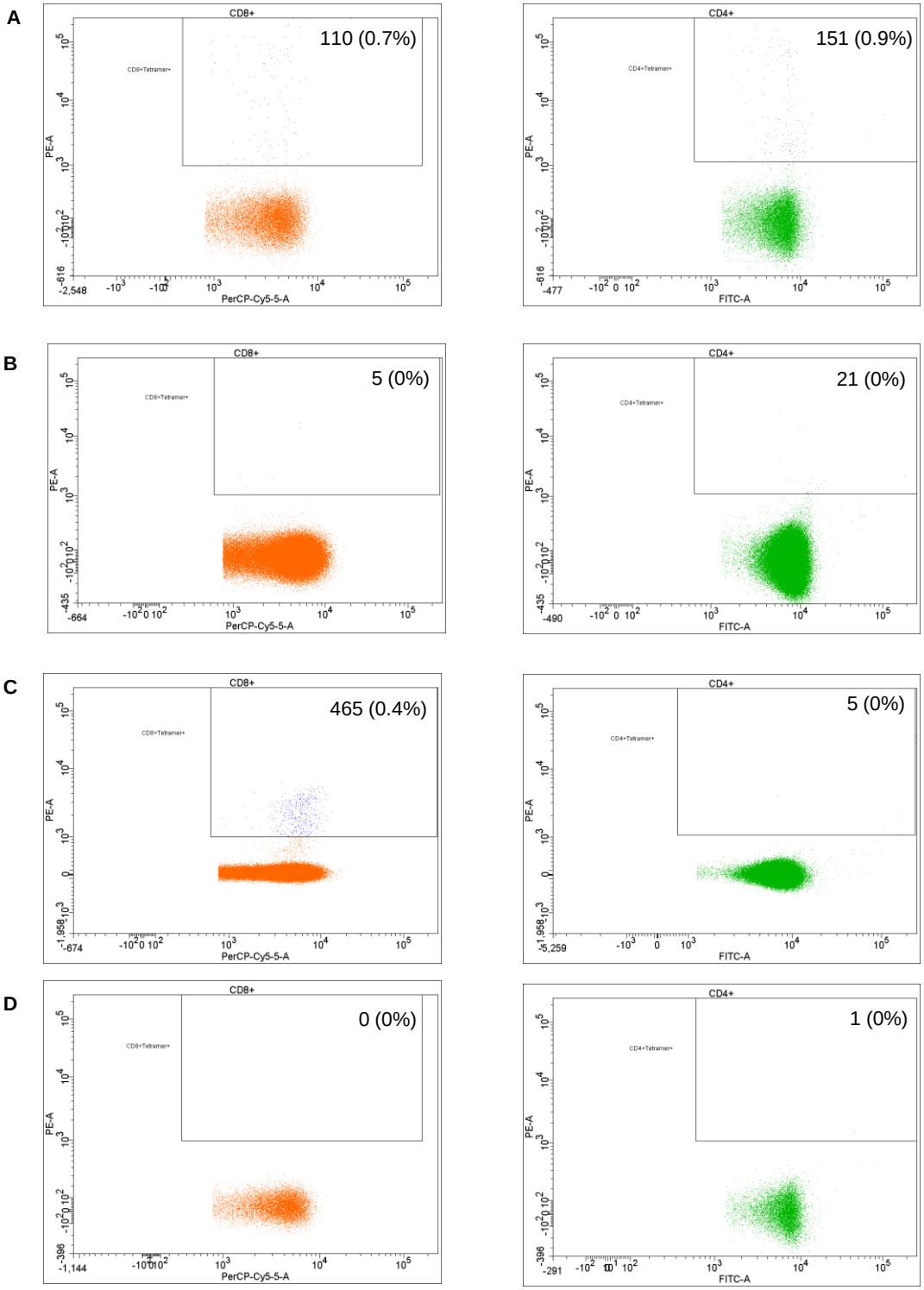


Figure 9-15: Representative flow cytometry findings for the detection of CTL response to PASD1 using a tetramer loaded with a PASD1 derived epitope. Gating was performed on live and singlet T cells. A: cancer patient PASD1 RT-PCR +ve PASD1 tetramer; B: cancer patient PASD1 RT-PCR +ve HIV tetramer; C: cancer patient PASD1 RT-PCR +ve EBV tetramer; D: Healthy patient PASD1 tetramer

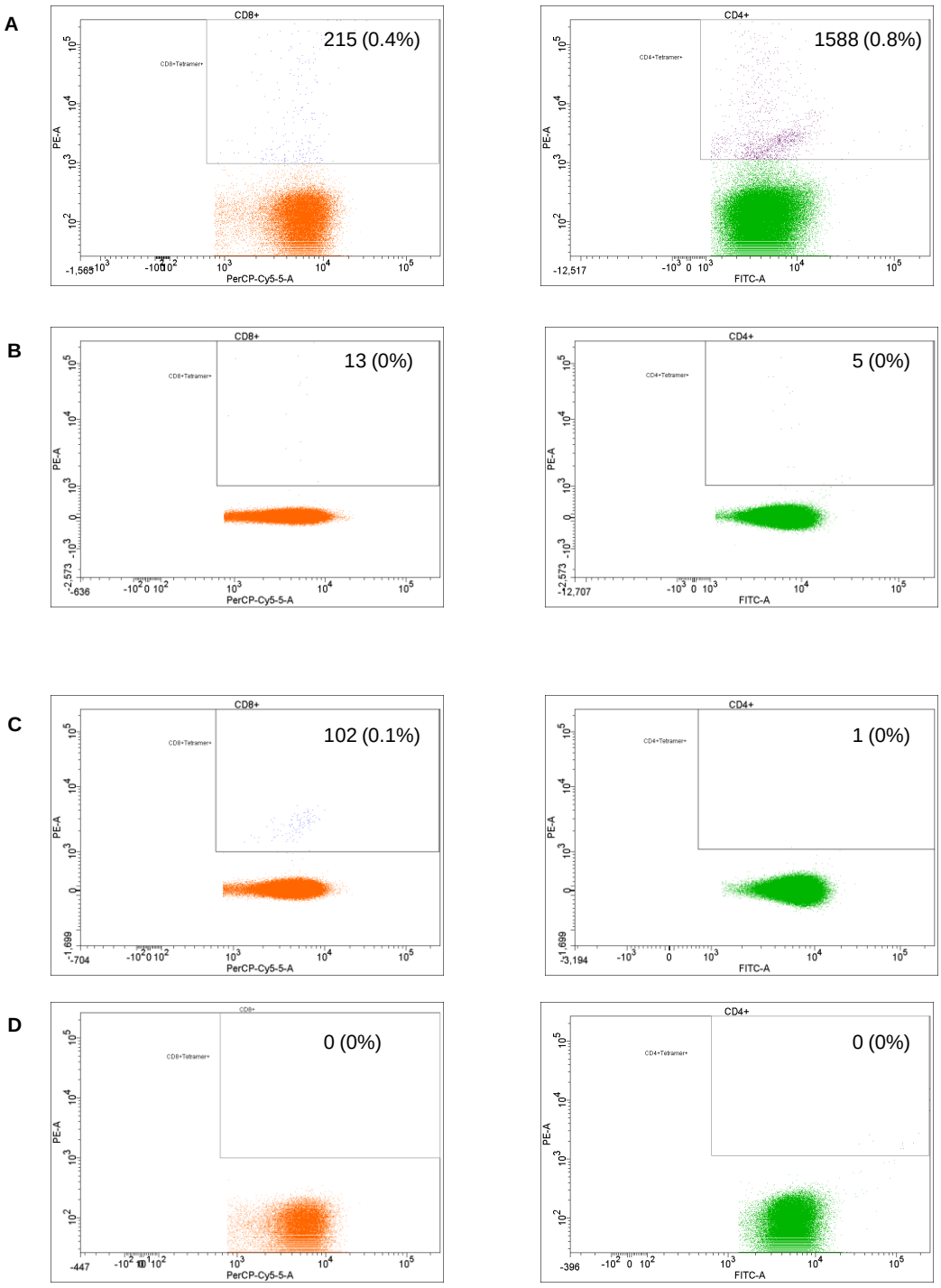


Figure 9-16: Representative flow cytometry findings for the detection of CTL response to CTAGE1 using a tetramer loaded with a CTAGE1 derived epitope. Gating was performed on live and singlet T cells. A: cancer patient CTAGE1 RT-PCR +ve CTAGE1 tetramer; B: cancer patient CTAGE1 RT-PCR +ve HIV tetramer; C: cancer patient CTAGE1 RT-PCR +ve EBV tetramer; D: Healthy patient CTAGE1 tetramer

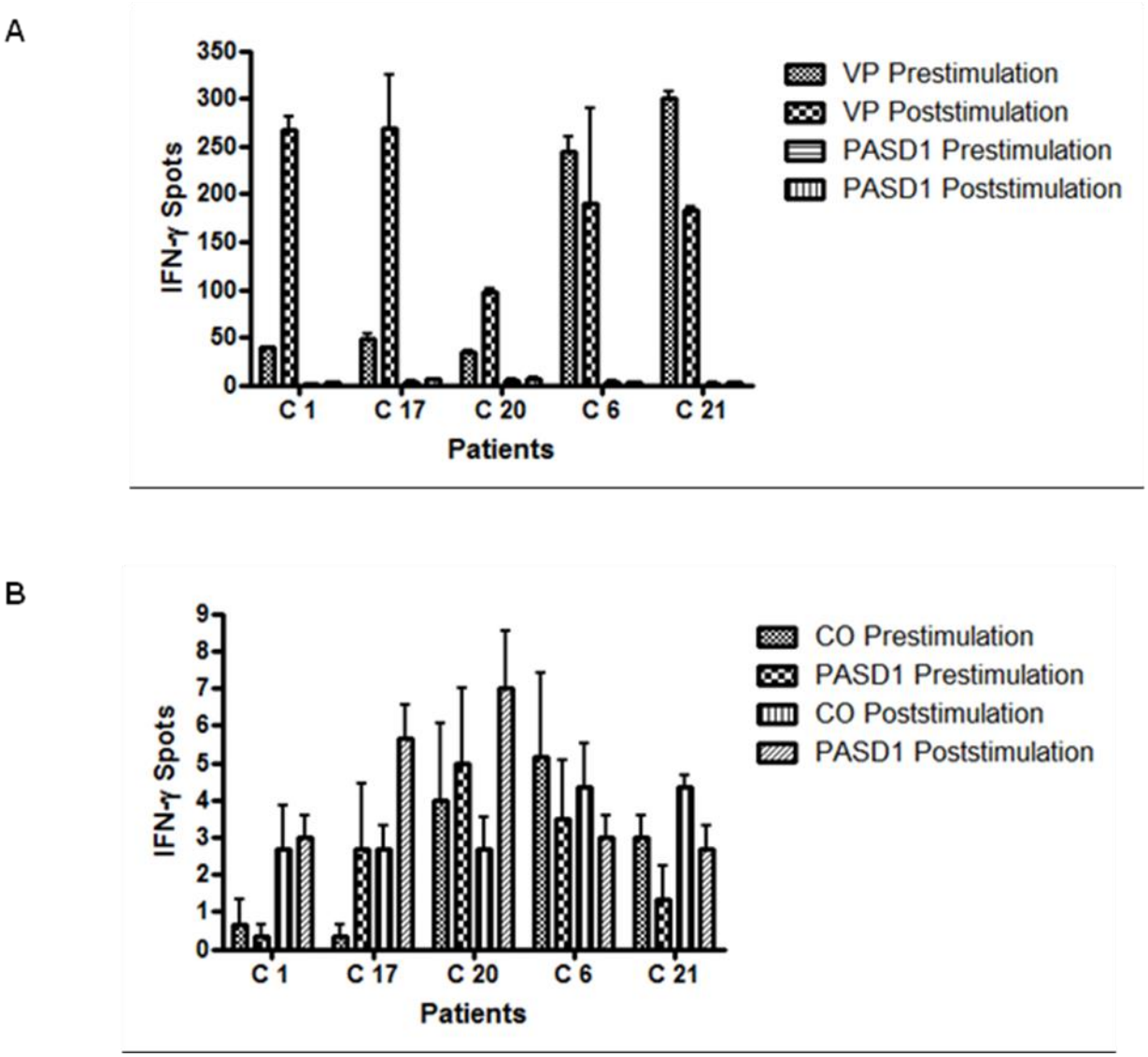


Figure 9-17: Representative ELISPOT findings for the detection of IFN-Ɣ production to PASD11 in cancer patients +ve for PASD1 by RT-PCR with and without stimulation. A: including positive controls (viral pool, VP); B: including negative controls only (cells only, CO)

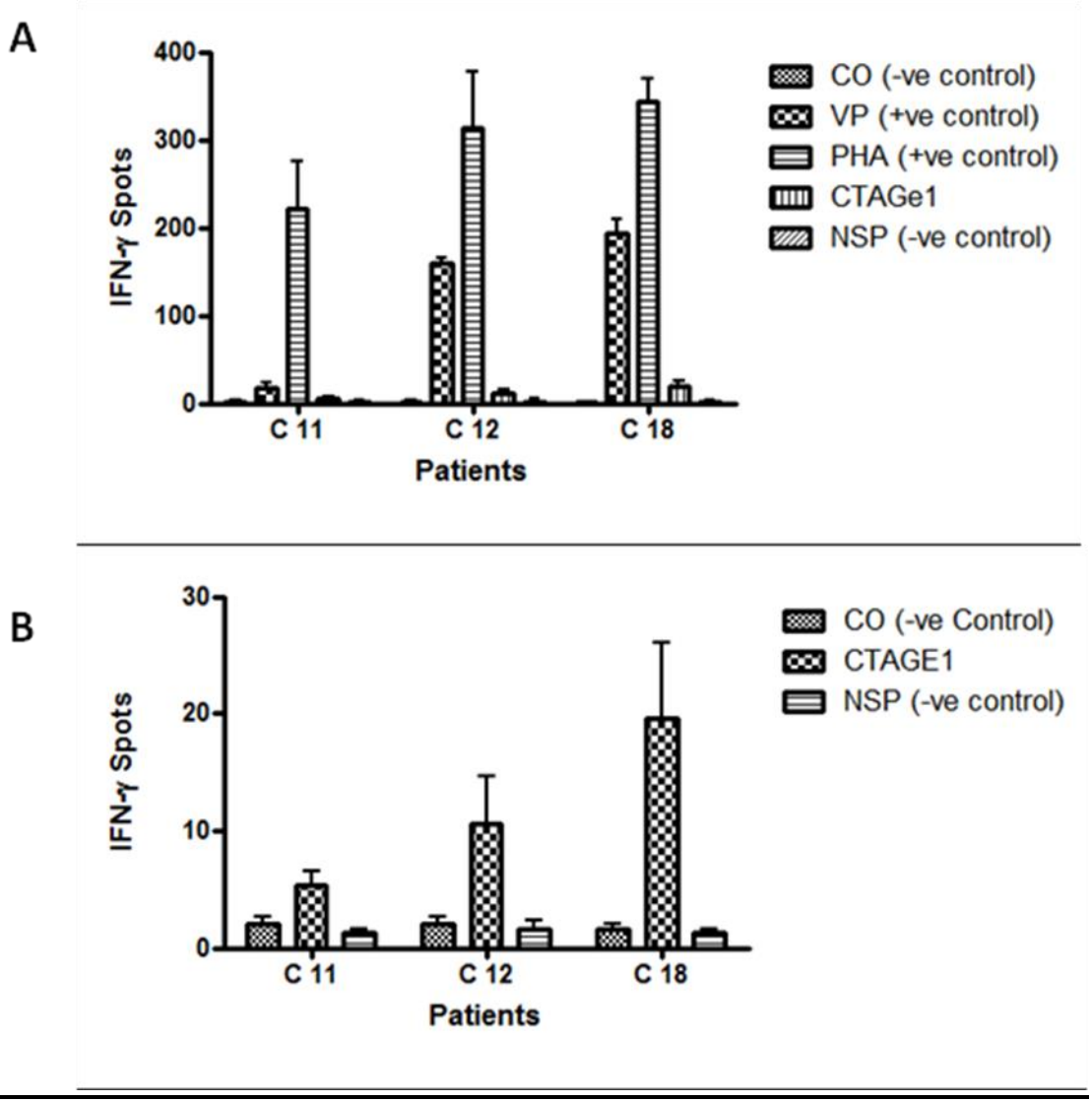


Figure 9-18: Representative ELISPOT findings for the detection of IFN-Ɣ production to CTAGE1 in cancer patients +ve for CTAGE1 by RT-PCR without stimulation. A: including positive controls (phytohemagglutin, PHA) and viral pool (VP) and –ve controls (Cells only, CO, and non-specific peptide, NSP); B: including negative controls only (CO and NSP)

9.4. Discussion

The radical treatment options for patients with OAC remain limited to resection of the tumour with or without additional chemotherapy and/or radiotherapy. This current approach provides modest survival rates. Furthermore, the majority of patients present with advanced disease and often are not amenable to potentially curative treatment. We have therefore studied CTAs for their potential clinical uses, as immunotherapeutic targets or biomarkers, for these patients who are difficult to treat.

In this study we found broad expression of CTAs, both via gene expression and at the protein level, via immunohistochemistry and serological autoantibodies, in BO with increasing expression in OAC compared with limited expression in normal oesophageal tissue. There was also a significant increase in expression from the premalignant condition, BO, to invasive OAC. In addition there was increasing gene expression of CTAs with stage of disease although this did not reach statistical significance. These findings in OAC are in keeping with other studies on solid tumours including OSCC that have found broad expression as well as increasing expression with stage of disease. The rationale for broad expression and increasing expression of CTAs with stage of disease is likely to represent the induction of CTAs being caused by random promoter demethylation, a process which noticeably increases with disease progression.

The finding of CTAs in BO patients is of particular note and calls into question its premalignant status. It has been suggested that BO is in fact neoplastic due its clonality and hyperproliferative nature, as well as the increased risk of progressing to invasive cancer it confers (541-543). We have shown that CTA expression is intraclonal in oesophageal cell lines and this supports these findings. Perhaps the identification of CTAs in BO is not so unexpected given these findings and the additional observation by Smith *et al* that DNA methylation is postulated to be caused by reflux of gastroduodenal contents. Aberrant DNA methylation was observed to fall in patients after successful fundoplication compared to BO who have remained on sole medical treatment (544). The pattern of CTA expression in BO, with limited expression in normal oesophageal tissue, suggests that CTAs could be ideal biomarkers of progression from BO to OAC with a potential functional role in early tumourogenesis of OAC (545).

In keeping with other studies we found significant co-expression of CTAs within tumours as well as heterogenous expression between tumour cells. The co-expression observed in this study as well as other studies indicates that CTA expression may result from the loss of DNA methylation. MAGEA3 has been suggested to be a good marker for overall gene activation of CTAs and we observed this as the patients with MAGEA3 gene expression expressed further 6 CTA genes (546). However, to analyse the specificity of a particular CTA we found to be highly expressed in BO and OAC we analysed the expression of CTA genes around the PASD1 gene locus. We found only 20% co-expression of FATE1 and PASD1 in OAC patients tumour which suggests the selective

induction of PASD1 in this condition. This, taken with its high expression in BO with increasing expression in OAC, suggests its potential role in the progression of BO to OAC.

For immunotherapeutic targets, genes with the most restricted expression pattern in normal tissue, testis restricted or testis/brain restricted, will be the most attractive. Minimal expression of CTAs was observed in normal oesophageal tissue whether from normal tissue from a patient with OAC or from a healthy patient with non-specific upper gastrointestinal symptoms. A number of testis selective CTAs had no expression in normal tissue and so are not necessarily precluded from being used as a immunotherapeutic target. These findings offer the potential for a single CTA to act as a target for a high percentage of patients. In addition the approach used in this study offers the ability to identify targets on an individualised basis. CTAs could be utilised as either a single or multiple antigen delivered vaccine to boost the immune response.

The confirmation of protein expression of CTAs in OAC adds to their suppression profile. The association of worse DFS and NY-ESO1 IHC staining highlights a group of patients who would benefit from adjuvant novel therapies. NY-ESO1 is highly immunogenic and so this group of patients may benefit from vaccine directed towards this TAA and a personalised approach would be required given the low frequency of expression seen for individual CTAs.

To develop a cancer-specific immunotherapy, peptide epitopes capable of inducing an immune response are required. To this end we sort to identify epitopes from CTAs expressed in OAC. To date we have not been able to show any specific CD8+ positive response from PBMCs from patients with OAC to peptides derived from CTAs. Two of three patients showed IFN-Ɣ response to CTAGE1. These responses, when taken with responses against CTAs identified by other groups, suggest immunogenicity towards CTAs in OAC patients. To establish the immunogenicity of CTAs that we have found to have high frequency of gene expression in BO and OAC, further experiments will be required. These will include looking at different CTA epitopes, assessment of Class 1 expression on CD4 cells and assessing the observed response in patients with cancer but not expressing the CTA of interest.

To assess their biomarker potential enzyme-linked immunosorbent assay (ELISA) was performed on patient serum to detect autoantibodies specific to select CTAs and helps to confirm their immunogenicity. We have shown the potential usefulness of a novel serum assay not only to show CTAs as potential biomarkers of disease progression but also as away to screen for potential immunotherapeutic targets.

The study is not without limitations and although statistically significant, the numbers are relatively small and cross-validation in preferably prospective cohorts will be required. However the study was efficient in its approach with both RT-PCR and qRT-PCR being used to assess gene expression in addition to confirmation of protein expression on tissue microarrays. Further analysis by means of antigen-specific CTL

response further substantiates these findings as well as offers confirmation of the ability of CTAs to be used as an immunotherapeutic target.

This study suggests some potential translational benefits. Firstly CTAs can offer a therapeutic target to induce, and boost, a cancer specific immune response. The observation that a spontaneous immune response observed in solid tumours is associated with improved long-term outcome has heightened interest in boosting this response to prevent tumour recurrence (227). CTAs are ideally suited to act as the target due to their restricted expression pattern to cancer or immunoprivileged sites such as the brain or testis. In addition the confirmation of their ability to induce an immune response both humoral and cellular, to lyse tumour cells, furthers their attractiveness as targets (547, 548). This study suggests that this may well require personalisation due to no individual CTA having expression in all tumours. However an approach of screening patients, either by immunohistochemistry or serum autoantibodies for targeted CTAs followed by selecting appropriate immunogenic epitopes through tetramer assays, offers a platform to take this into clinical practice.

Secondly CTAs offer potential as biomarkers of disease progression from BO to OAC in highlighting subclinical recurrence in post resection patients. Their potential use is emphasised in that CTAs have increased expression: with stage of disease in solid cancers; in mesenchymal and human embryonic stem cells and in other transformed or premalignant conditions. These findings suggest that CTAs have a role in cancer cell development and cell differentiation (537-539). The ability to identify patients at an early stage of tumour development is likely to have the greatest impact on survival as patients with stage I disease have excellent 5 year survival rates of greater than 95% with current treatment approaches. Alternatively they may serve to stratify patients with BO, HGD and OAC to better guide patient management decisions along more radical or conservative treatment or surveillance pathways dependent on their expression profile.

In conclusion, in this study we for the first time describe the identification of CTA genes in BO and provide the first wide screen of CTAs in OAC. This provides an efficient and relatively inexpensive method to screen patients for tumour antigen gene expression to identify patient groups and individual patients suitable for immunotherapy. The identification of CTAs in BO suggests their involvement in early OAC tumourgenesis and offers the potential for CTAs to be clinically useful biomarkers of disease progression in addition to being attractive immunotherapeutic targets.

10. Chapter 10: General summary and discussion

10.1. General Discussion

This thesis has described strategies to improve outcomes for patients with OAC. The thesis is split in two parts firstly assessing recent innovations that serve to improve on perioperative care either by improved staging, surgical technique or postoperative care. The second part of the thesis offers potential strategies to provide targets for immunotherapy and biomarkers of prognosis, progression and response to neoadjuvant chemotherapy. Much of the discussion of the work in this thesis is presented in the preceding chapters. This chapter discusses the findings in a wider context, with reference to the field and areas for future research.

Use of PET/CT in routine clinical practice in the UK had been variable and dependent largely on availability of this imaging modality. It was felt the use of PET/CT was likely to add accuracy over PET alone by enabling morphological and metabolic information to be gained in one sitting allowing more precise interpretation. PET imaging not combined with CT had been demonstrated in a multi-centre randomised trial in America to identify inoperable metasteses in an additional 5% of patients compared to conventional staging (549). Despite the variable introduction of PET/CT in the UK none had identified its use in a multi-centre setting and we demonstrated, in a multicentre observational study, its use in detecting additional metasteses 9%. We have confirmed the role of PET/CT in a multicentre setting in detecting distant metastases not detected by conventional staging thus provding more informed treatment choices for these patients. In clinical practice patients undergo palliative treatment rather than undergo the risks of a curative regime.

The most important role of PET/CT potentially lies in reducing the chance of early recurrence after curative resection. This role is not yet confirmed and it is essential to carefully follow up this and other cohorts of patients having had PET/CT, in order to define the impact of PET/CT on early recurrence of disease. In addition where PET/CT sits in the preoperative staging algorithm has yet to be defined as it may be useful to highlight patients that require no further work up such as CT and EUS and if used later in preoperative staging its use may not be required in patients with disease defined as early stage by CT and EUS.

With regard to surgical techniques minimally invasive oesophagectomy had re-emerged in a variety of forms. The goal of minimally invasive surgical techniques is to reproduce the open operation in a format that reduces surgical trauma and improve patient outcomes. However despite increasing reports of the benefits of minimally invasive oesophagectomy (128) none had compared minimally invasive two stage oesophagectomy (MIO-2) with open Ivor Lewis oesophagectomy, the preferred operation in the UK, its open counterpart. We have demonstrated that MIO is without detriment when compared directly with IVL for all measured parameters in a single centre. MIO-2

can be introduced safely within the context of a specialist team and future trials (550) including quality of life as end-points are ongoing to assess the true benefits of MIO-2.

Despite these potential improvements in preoperative staging and surgical technique morbidity and mortality occur in significant numbers following oesophageal resection. We therefore sought to identify a method to identify patients who develop these serious complications before their symptoms develop as rapid diagnosis of AL and major complications is essential to limit contamination and minimise sepsis (158) as this may lead to improved clinical outcomes both in the short and long term. Whilst many clinicians use markers of the systemic inflammatory response to monitor a patient's post-operative course little is known of their predictive accuracy. In addition we hypothesised that we may be able to develop a novel score by combining these markers to increase diagnostic accuracy. We have buillt on previous reports to highlight the efficacy of blood borne markers of the systemic inflammatory response as predictors of major complications following upper gastrointestinal surgery (174, 179) and highlighted the utility of combining these markers to increase diagnostic accuracy. An assessment of the utility of the score in a prospective setting is required. In addition alternative markers of the systemic inflammatory response may improve diagnostic accuracy and increase biological understanding so this inflammatory response can potentially be modulated to improve outcomes.

To enable future work to assess immunological markers to identify responders to neoadjuvant and novel immunotherapies we sought to identify what constitutes a significant pathological response to neoadjuvant chemotherapy. A robust and accurate assessmet of pathological response to neoadjuvant therapy is fundamental to guiding selection of tailored pre and post operative treatment strategies, identify biomarkers of response to chemotherapy, provide prognostic information and assess neoadjuvant therapies. TRG is the proposed method of assessing pathological response in OAC following neoadjuvant chemotherapy despite being defined in OSCC patients after having received neoadjuvant chemoradiotherapy (83). We propose that methods to assess the pathological response to NAC are refined so that both the response in the primary tumour and the regional lymph nodes are considered as we showed both responses in the tumour and the lymph nodes were independently associated with survival. This will require further validation preferably in a prospective setting. It was clear from our study that some patients with modest or no tumour response and lymph node downstaging have improved survival compared to patients with poor response in both the primary tumour and lymph nodes. This identifies a previously unrecognised group of patients who may benefit from further postoperative therapy. These findings will require assessment following neoadjuvant chemoradiotherapy in previous cohorts (187) and future trials (551).

One can now begin to consider an algorithm for perioperative treatment of OAC that may involve induction therapy followed by early identification of responders and the

curtailment of, or a change to, neoadjuvant therapy for non-responders. Further analysis of the primary tumour and lymph nodes after surgery would direct patients with poor tumour response, to NAC, but nodal downstaging to adjuvant therapy. This kind of stratified therapy will be supported by ongoing studies of biomarkers. The contribution of the host systemic inflammatory response and tumour microenvironment is also likely to offer new targets for therapy and may be the place to look to explain the different responses to therapy observed between otherwise similar tumours.

We therefore proceeded to initially investigate the host immunological response and we confirmed that biochemical markers of nutritional status and the systemic inflammatory response are independent predictors of survival in patients undergoing radical treatment for oesophagogastric cancer. We also highlighted that malnutrition is common preoperatively and that hypoalbuminaemia prior to the start of chemotherapy predicted response to chemotherapy. This has potential treatment implications as pretreatment nutritional status and increased systemic inflammation are potentially modifiable interacting risk factors. For instance, it may be possible to target treatments to patients during neo-adjuvant chemotherapy or during the perioperative period (514-516). A recent review of immunonutrition in patients undergoing oesophageal cancer resection has called for well designed randomized controlled trials to assess the potential benefits of immunonutrition targeted to the nutritionally compromised (517). Whilst these strategies will require prospective trials over long periods that have now been started, the more immediate impact of this study maybe to better risk-stratify patients by incorporating available biochemical markers of systemic inflammation and nutritional status into current preoperative risk assessment tools.

To follow on from assessing the systemic inflammatory response we went on to assess the local immune response by concentrating on TILs. We obtained three major findings: firstly, we have shown associations between pathological stage of disease and TIL density and confirmed the independent association of particular TIL subsets and survival; secondly we have shown significant correlation between TIL subtypes in OAC, and finally we found patients with a significantly increased pathological response to neoadjuvant chemotherapy had higher levels of TILs in their resected tumour, most notably with CD4+ and CD8+ TILs for local tumour regression and lymph node response respectively. These findings give rise to potential clinical applications. This TIL response could be used in the selection of patients for specific adjuvant therapies. A significant response to neoadjuvant chemotherapy was associated with higher infiltration with TILs post therapy. Further work analyzing the function of these TIL subsets and the correlation of pretreatment TIL densities with response to therapy may unearth predictors of response to chemotherapy in addition to aid design of novel treatments or as a mechanism to improve response to current therapies.

We then sought to identify a strategy to harness the immune response and choose to assess tumour associated antigens that could act as targets for the adaptive immune response. We for the first time describe the identification of CTA genes in BO and provide the first wide screen of CTAs in OAC. This provides an efficient and relatively inexpensive method to screen patients for tumour antigen gene expression to identify patient groups and individual patients suitable for immunotherapy. The identification of CTAs in BO suggests their involvement in early OAC tumourgenesis and offers the potential for CTAs to be clinically useful biomarkers of disease progression in addition to being attractive immunotherapeutic targets.

10.2. Summary of findings and Future Directions

1. By evaluating the use of PET/CT in the first UK multicentre study we have confirmed its role in detecting unsuspected metastases in preoperative staging amongst patients thought, on the basis of CT/EUS, to be candidates for curative treatment.

Future work: The most important role of PET/CT potentially lies in reducing the chance of early recurrence post resection and the reporting of longer term outcomes may confirm these short term findings.

2. The direct prospective comparison of MIO-2 with its open counterpart the IVL oesophagectomy in a single centre setting has demonstrated that MIO-2 is without detriment when compared directly with IVL for all measured oncological and surgical outcomes. MIO can be introduced safely within the context of a specialist team. However significant benefits of MIO-2 over IVL were not revealed.

Future work: Studies comparing MIO-2 with open IVL oesophagectomy with quality of life related data as the primary end-point and cost-effectiveness as a secondary analysis would be the logical next step to potentially reveal any true benefits of MIO, preferably within the setting of a randomised control trial.

3. We have highlighted the efficacy of blood borne markers of the systemic inflammatory response as predictors of major complications following upper gastrointestinal surgery and have developed a novel score which combines these markers to predict AL with 95% sensitivity and high diagnostic accuracy.

Future work: Studies developing scoring systems require cross-validation in other similar patient cohorts to assess its true validity. To confirm the potential of its clinical use in reducing the use of blood tests postoperatively, predicting major complications early and as an adjunct to a protocol driven postoperative treatment algorithm, an assessment of the utility of the score in a prospective setting is required. In addition alternative markers of the systemic inflammatory response may improve diagnostic accuracy and increase biological understanding so this inflammatory response can be modulated to improve outcomes.

4. A) We have confirmed that a significant pathological response as described by *Mandard et al* is associated with improved DFS.

   B) Secondly we have confirmed that lymph node downstaging by neoadjuvant chemotherapy leads to improved DFS.

   C) We propose that methods to assess the pathological response to NAC are refined so that both the response in the primary tumour and the regional lymph nodes are considered

Future work: A robust and accurate assessmet of pathological response to neoadjuvant therapy is fundamental to guiding selection of tailored pre and post operative treatment strategies, identify biomarkers of response to chemotherapy, provide prognostic information and assess neoadjuvant therapies. This will enable future work to assess immunological markers and molecular imaging to identify responders to neoadjuvant and novel immunotherapies.

5. A) We have confirmed the association between routinely used blood borne markers of nutritional status and the systemic inflammatory response with survival in patients with OAC who have undergone radical treatment.

   B) Secondly, we found malnutrition, as measured by PNI, to be both common preoperatively and to show an inverse association with the systemic inflammatory response.

   C) We found that hypoalbuminemia, before the start of chemotherapy, was associated with a lack of pathological response to neoadjuvant chemotherapy.

Future work: A trial to assess whether nutritional or immunonuritional supplementation during multimodal treatment for oesophageal cancer can improve tumour response to neoadjuvant therapy, reduce pos-operative complications and improve survival by modulating systemic inflammation. The more immediate impact of this study may be to better risk-stratify patients by incorporating NLR, albumin and PNI into current preoperative risk assessment tools. In addition, it would be pertinent to assess the

modulation of these markers after the 1st cycle of neoadjuvant therapy for the predictive value of response to neoadjuvant therapy.

6. A) We have shown associations between TILs and pathological stage and confirmed the association of TILs and survival.
   B) Secondly we found significant correlation between TILs.
   C) We found higher levels of TILs postoperatively to be associated with a significantly increased pathological response, most notably with CD8+ TILs.

Future work: To analyse any correlation with known immunosuppressive markers to understand why there is a reduction of tumour infiltrating lymphocytes and identify the function of intratumoural lymphocytes in OAC. This will lead to an increased understanding of the tumour biology to identify potential therapeutic strategies.

7. A) We have identified broad expression of CTA gene transcript expression in BO patients with increasing expression in OAC patients with limited frequency of CTA expression in normal oesophageal tissue. These findings make CTAs attractive immunotherapeutic targerts in OAC and BO as well as potential biomarkers of progression.
   B) We have identified a potentially immunogenic epitope derived from CTAGE1.
   C) The high frequency of expression of CTAs in BO and OAC make them potential biomarkers of progression. They may also play a role in the pathogenesis.
   D) A novel immunological assay to detect autoantibodies for CTAs enables a relatively non-invasive means to detect CTAs in patients at different stages of disease. Utility could include detection of progression from BO to OAC, recurrence after conventional treatment and selection for personalised novel immunotherapies.

Future work: To confirm these findings cross-validation in other preferably prospective cohorts would be beneficial. To confirm the ability of select CTAs to produce an immunogenic response in OAC patient's further epitopes would need to be assessed for CTL responses. In addition functional experiments to increase the understanding of these antigens would be of benefit prior to them being utilised as a target.

10.3. Conclusion

We have confirmed the utility of positron emission tomography - computed tomography in staging by observing its use in a multi-centre United Kingdom setting and

found an additional 9% of occult distant metastases compared to traditional staging methods, justifing its use by reducing the radical treatment of patients with metastatic disease.

Minimally invasive oesophagectomy has been recently introduced into practice in a few specialist centres and we compared minimally invasive with open Ivor Lewis oesophagectomy showing there to be no detriment when compared directly for short and medium term outcomes.

With regard to improving morbidity we developed and prospectively validated a novel scoring system, based on markers of the systemic inflammatory response, to predict major complications and anastomotic leak earlier than standard postoperative care.

Both neoadjuvant chemotherapy and oesophagectomy are associated with significant morbidity. To improve outcomes further we firstly define what represents a significant immunopathological response to neoadjuvant chemotherapy, suggesting that both the response in the tumour and lymph node is adopted as a method to evaluate tumour regression, as it is these patients that have a significant benefit. We highlight immunonutritional blood-borne markers that predict both long-term survival and response to neoadjuvant chemotherapy.

In terms of novel treatments for oesophageal cancer, immunotherapy remains attractive. Current immunotherapies have not delivered significant results in solid tumours, the reasons for this being multifactorial but include the ability of the tumour to evade the immune response. We define the local tumour inflammatory environment and specific target tumour antigens, cancer testis antigens, as potential cancer vaccine targets and biomarkers in oesophageal adenocarcinoma.

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12. Chapter 12: Appendices

12.1. Appendix 1: Overview of study plan for tissue storage

Table 12-1: Tissue storage plan

SAMPLES					Storage Method				
Time point	Sample number	Sample Expected	Volume expected (mL)	Receipt, Process, Logging, Labelling	Serum	PBMC	Snap Frozen	Resin Embedding	Available from Pathology
Cohort 1 Endoscopy:									
Endoscopy Day	01	Clotted blood	0-50			■	■	■	■
	02	EDTA blood	0-50		■		■	■	■
Endoscopy Biopsy	03	Tissue	1		■	■			■
Cohort 2: Surgery									
Pre-op	04	Clotted blood	0-50			■	■	■	■
	05	EDTA blood	0-50		■		■	■	■
Operation Day	06	Tumour Tissue	5+		■	■			■
Operation Day	07	Barrett's	5+		■	■			■
Operation Day	08	Normal Oesophagus	10+		■	■			■
Operation Day	09	Normal Stomach	10+		■	■			■
Follow-up 6 Weeks	10	Clotted blood	0-50			■	■	■	■
	11	EDTA blood	0-50		■		■	■	■
Cohort 3: Chemotherapy									
Endoscopy Biopsy	12	Parrafin Embedded	0-50		■	■	■		
Pre-chemotherapy (Baseline)	13	Clotted blood	0-50			■	■	■	■
	14	EDTA blood	0-50		■		■	■	■
Post-chemotherapy	15	Clotted blood	0-50			■	■	■	■
	16	EDTA blood	0-50		■		■	■	■
Cohort 4: Radiotherapy									
Endoscopy Biopsy	17	Parrafin Embedded	1		■	■	■		
pre-radiotherapy (Baseline)	18	Clotted blood	0-50			■	■	■	■
	19	EDTA blood	0-50		■		■	■	■
Post-radiotherapy	20	Clotted blood	0-50			■	■	■	■
	21	EDTA blood	0-50		■		■	■	■
Cohort 5 Historical:									
Endoscopy biopsy	22	Paraffin Embedded	1		■	■	■	■	
Resection biopsy	23	Paraffin Embedded	1		■	■	■	■	

Notes for table 12-1

The clear cells indicate the availability and how samples are to be stored.

Special Considerations for Endoscopic and Operative Specimens

Endoscopic Samples:

Endoscopic biopsies may be taken from normal oesophagus, normal stomach, Barrett's or areas of carcinoma. Due to the heterogeneity of dysplasia within areas of Barrett's metaplasia and presumed cancer it is important to have accurate histopathological assessment of the biopsy specimens. Therefore each biopsy from an area of suspected Barrett's or Barrett's associated cancer will be divided in the endoscopy suite by tissue bank staff or the endoscopist. Half the sample will be snap frozen for tissue banking and the other half sent for routine histological assessment. Both the tissue bank sample and the path sample will be labelled with the same number as follows:

Barrett's oesophagus: B1, B2, B3 etc

Cancer: C1, C2, C3 etc

So as to reduce waste the samples being sent for routine histological assessment will be placed on filter paper and labelled as above. This is the same as for mapping biopsies at colonoscopy.

Normal oesophagus and stomach biopsies will not be halved and will be labelled: O1, O2, O3 and S1, S2, S3 respectively.

Therefore a representative final tissue bank code would read:

Patient No.-Sample code No.-Specimen No.

i.e 001-03-S1 (Patient 1-Endoscopy biopsy-Normal Stomach 1)

Operative Specimens:

These will be labelled in a similar way but not halved.

12.2. Appendix 2: Patient information sheet example

CANCER RESEARCH UK

Southampton NHS University Hospitals NHS Trust

UNIVERSITY OF Southampton School of Medicine

Cancer Research UK Clinical Centre
Somers Cancer Research Building
Mail Point 824
Southampton General Hospital
Tremona Road
Southampton SO16 6YD, UK

T 023 8079 6184
F 023 8079 5152

Director Professor Peter Johnson

PATIENT INFORMATION SHEET - Surgery

Study Title: **Oesophagus: molecular, cellular and immunological assessment**
REC No.: **09/H0504/66**

1. Introduction

You are being invited to take part in a research study. Before you decide it is important for you to 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 and your GP 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.

2. What is the purpose of this study?

Tumours of the oesophagus (the gullet; a tube connecting mouth and stomach) and stomach are relatively common in our population and are obviously a serious health issue. You have been referred to Southampton University Hospitals NHS Trust as it is a regional specialist centre for the treatment of this group of cancers and has a group of experts for this condition; and is why you are having your operation here.

We are always looking to improve on the treatments we provide. To do this we need to understand more about these cancers; particularly why they occur and what promotes their development. This greater understanding may permit us to develop improved treatments in the future.

The research we wish to undertake will be conducted by the clinical team involved in your care and by research scientists in the University of Southampton. The research may involve studying tissue samples from patients like yourself who are undergoing treatment for cancer. These samples may be examined in detail for the molecular genetic changes that have occurred for cancer to develop and also for the body's own immune response to the cancer. This research may involve the analysis of DNA that may lead to the discovery of genes that indicate an increased risk of oesophago-gastric cancer for you or your relatives. These investigations may aid our discovery of mechanisms to help treat oesophago-gastric cancer in the future.

3. Why have I been chosen?

We are asking you to take part in our study because you have been diagnosed with cancer of the oesophagus or stomach and are undergoing treatment for this condition.

4. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

5. What will happen to me if I take part?

We will take a sample from your cancer after it has been removed by the surgeon at your operation.

We will take 50 ml of blood from you (this is about 10 teaspoons and is about one fifth of what a blood donor would give) before surgery (at the preadmission clinic approximately 2 weeks before surgery) and then again approximately three months after your surgery when you will be attending clinic for a routine follow up visit.

We would also seek not to waste any of your tissue and if there is excess we would like your permission for your tissue sample(s) to be used for future research. This is subject to the sample(s) being stored in the Human Tissue Authority (HTA)-licensed Tissue Bank in the Southampton Cancer Research UK Clinical Centre. The sample would then only be used in the future for research approved by the Tissue Bank management committee or on a future research project which has gained approval from a NHS Research Ethics Committee.

6. What are the side effects of taking part?

We will try and take the blood at the same time as other blood tests. If we cannot do this then it will be like any other blood test.

The clinical team looking after you will talk to you about the operation that you will have and they will explain the risks and benefits of the procedure in detail. There are no additional risks in donating tissue samples for this research as the samples will be taken from the surgical specimen after it has been removed during your operation.

7. What if there is a problem?

Complaints:
If you have any concerns or wish to complain about any aspect of the way you have been approached or treated as part of this study, you should initially contact the researcher, at the NHS Trust where you are being treated, who will do their best to answer your questions. The researchers contact details are provided at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your NHS Trust. Advice can also be sought from your local Patient Advice and Liaison Service (PALS), in person or by telephone. You can also contact the Research Governance Sponsor of this study, Southampton University Hospitals NHS Trust, R&D Department, MP 138, Southampton General Hospital, Tremona Road, Southampton SO16 6YD

Harm:
Every care will be taken to ensure your safety during the course of the study. Cancer Research UK Clinical Centre, the Research Governance Sponsor of the study has indemnity (insurance) arrangements in place for non-negligent harm, in the event that something does go wrong and you are harmed as a result of taking part in the research study. 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, or have concerns about any aspect of the way you have been approached or treated during the course of this study the normal National Health Service complaints mechanisms should be available to you.

8. What are the possible benefits of taking part?

There will be no direct benefit to you from taking part in this study. It is hoped the information we get from this study will help us develop better treatments for cancer.

9. Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept strictly confidential by your clinical carers and the research team. The handling, processing, storage and destruction of study information and data will conducted in accordance with the Data Protection Act 1998.

10. What will happen to the results of the research?

The results will hopefully be published in a reputable journal. You will not be identified in any report.

11. Who has ethically reviewed the project?

This Study has been reviewed by the local ethics committee.

12. Contact for further information:

You can contact:

Mr Tim Underwood, Clinical Lecturer in Surgery	023 8079 8632
Dr Andrew Bateman, Consultant Clinical Oncologist	023 8079 6184 (secretary)
Mr Fergus Noble, Clinical Research Fellow 023 80796670	

Thank you for taking the time to read this leaflet

12.3. Appendix 3: Patient consent form

CANCER RESEARCH UK

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton
School of Medicine

Cancer Research UK Clinical Centre
Somers Cancer Research Building
Mail Point 824
Southampton General Hospital
Tremona Road
Southampton SO16 6YD, UK

T 023 8079 6184
F 023 8079 5152

Director Professor Peter Johnson

Patient Identification Number for this trial: REC No.: 09/H0504/66

CONSENT FORM

Title of Project: **Oesophagus: molecular, cellular and immunological assessment**
Name of Researchers: **Mr Tim Underwood/ Dr Andrew Bateman/Mr Fergus Noble**

Please initial box

1. I confirm that I have read and understand the information sheet (Version 2 dated 13May 2009) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes and data collected may be looked at by individuals from the Cancer Research UK Clinical Centre (Southampton), or from regulatory authorities or from the NHS Trust where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐
5. I give permission for left-over samples to be stored and used for future ethically approved studies. ☐
6. I give permission for DNA analysis to be carried out. ☐
7. I give permission for information to be collected, stored, and used for research in a database held at the Cancer Research UK Clinical Centre (Southampton). ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes

Patient Consent Form - Version 2 – 13 May 2009