Prognostic and predictive markers in oesophageal cancer

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 2: The prognostic role of the circumferential resection margin status in patients with oesophageal cancer includes work published previously. The published work (*"Prognostic significance of cancer within 1mm of the circumferential resection margin in oesophageal cancer patients following neo-adjuvant chemotherapy"* was authored by Mr T Salih, Mr P Jose, Mr A Mirza, Mr S Mehta, Mr J Hayden, Dr SA Prichard, Dr G Udall and Dr H I Grabsch. The manuscript was published 2013 in the European Journal of Cardiothoracic surgery. The candidate was directly responsible for all the analysis published in this work as well as the writing of the final manuscript. Other authors were involved in data collection (Jose/Mirza), supervision of data collection (Prichard/Udall/Grabsch), review of histological slides (Prichard/Udall/Grabsch), and manuscript critiquing (Hayden/Mehta/Grabsch).

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ABSTRACT

The incidence of oesophageal cancer has increased in the United Kingdom over the past 30 years and is the fifth most common cause of cancer death. However, prognosis prediction and treatment decision for individual patients currently still rely only on the determination of the Tumour Node Metastasis (TNM) classification.

This thesis aimed to identify markers which can predict prognosis in OeC patients who were treated either by chemotherapy and surgery or surgery alone. It examined the prognostic value of the circumferential resection margin status, tumour cell density and the intratumoural immune cell infiltration. Data from Leeds and Manchester patients as well as from the international OE02 trial were used.

The results suggest that the presence of tumour cells either within 1mm or at the circumferential resection margin (CRM) is related to a significantly worse prognosis in univariate analyses. However, the CRM status was not an independent prognostic marker for cancer specific survival after neoadjuvant chemotherapy and surgery.

Tumour cell density (TCD) was measured using well established morphometric methods at the luminal surface of the resected tumour in the OE02 cohort. A significant difference was seen in TCD between the two treatment groups. A three tiered TCD classification was found to be related to prognosis in the chemotherapy and surgery treatment group. However TCD was not an independent predictor of patient survival when established clinicopathological variables were included in the multivariate analysis.

Differences were found in the proportion of tumour infiltrating immune cells between the two treatment arms of the OE02 patients. The level of T cell infiltration was shown to have prognostic value in the chemotherapy followed by surgery treatment arm, although this was not an independent prognostic marker. When using a ratio score of T cell infiltration and macrophage infiltration at the tumour an independent prognostic marker is identified.

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I. ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ASR	Age Standardised Rate
AW	Mr Alex Wright
CAP	College of American Pathologists
cm	Centimetre
CRM	Circumferential Resection Margin
CS	Chemotherapy followed by Surgery group
CT	Computerised Tomography scan
DT	Dr Darren Treanor (Leeds Consultant histopathologist)
EU	European Union
EUROCARE	European Cancer Registry
EUS	Endoscopic Ultrasound Scan
FDG	Fluorine-18 Fluorodeoxyglucose
FOXP3	Forkhead Box Protein P3
GH	Gordon Hutchins (Leeds histopathologist)
GORD	Gastro-Oesophageal Reflux Disease
H&E	Haematoxylin & Eosin
HG	Professor Heike I Grabsch (lead research supervisor)
HPV	Human Papilloma Virus
IHC	Immunohistochemistry
JCEC	Japanese Classification of Esophageal Cancer
LICAP	Leeds Institute of Cancer and Pathology (formerly LIMM)
LIMM	Leeds Institute Molecular Medicine
LTHT	Leeds Teaching Hospital Trust
LW	Mrs Lindsay C Hewitt (senior laboratory technician)
M	distant metastasis category (as part of the TNM classification)

ml	Millilitre
mm	Millimetre
MRC	Medical Research Council
N	Lymph node category (as part of the TNM classification)
NCCN	National Comprehensive Cancer Network
NLR	Neutrophil to lymphocyte ratio (haematological measure)
NOGCA	National Oesophagogastric Cancer Audit
NW	Dr Nick West (Leeds histopathologist)
OeC	Oesophageal Cancer
OE02	Oesophageal cancer trial
PET	Positron Emission Tomography
POPP	Percentage of Positive Pixels
RCT	Randomised Controlled Trial
RECIST	Response Evaluation Criteria in Solid Tumours
RCPath	Royal College of Pathologists
RTOG	Radiation Therapy Oncology Group
T	Depth of invasion of the primary tumour category (as part of the TNM classification)
TAM	Tumour Associated Macrophages
TCD	Tumour Cell Density
TIL	Tumour Infiltrating Lymphocytes
TIN	Tumour Infiltrating Neutrophils
TMA	Tissue Microarray
TNM	Tumour Nodes Metastases classification
TRG	Tumour Regression Grade
TRG1	Complete regression showing absence of histologically identifiable residual cancer and fibrosis extending through the different layers of the oesophageal wall, with or without granuloma
TRG2	Characterised by the presence of rare residual cancer cells scattered through the fibrosis

TRG3	Involves an increase number of residual cancer cells but fibrosis still predominant
TRG4	Residual cancer outgrowing fibrosis
TRG5	Characterised by the absence of any regression changes
TSR	Tumour Stroma Ratio
UICC	International Union Cancer Committee
UK	United Kingdom
USA	United States of America
USS	Ultrasound Scan
WECC	Worldwide Oesophageal Cancer Collaborative
WHO	World Health Organisation

1. INTRODUCTION

This thesis will assess potential new prognostic markers for aiding in the management of oesophageal cancer patients and compare their relationship with markers currently used in the United Kingdom (UK). Oesophageal cancer incidence is increasing worldwide and most rapidly in the UK. Currently, the only established prognostic marker for OeC patients is the tumour node metastasis (TNM) classification.

This thesis aims to investigate whether the use of morphometrically measured tumour cell density of the primary tumour or immunohistochemical staining for intratumoural immune cells can be used to better stratified patients into treatment relevant prognostic groups improving and individualising post-operative patient management. This thesis will also examine the value of the post-operative circumferential resection margin clearance and compare the prognostic value of the presence of tumour cells at the CRM versus within a distance of one millimetre (mm).

1.1 Anatomy of the oesophagus

The oesophagus is a tubular muscular structure which connects the oral cavity with the stomach (Figure 1). The oesophagus begins at the pharynx at the level of the cricoid cartilage (around vertebral level C6) where the upper oesophageal sphincter is located. The oesophagus then extends caudally behind the trachea and thyroid gland, being located in front of the cervical vertebrae of the neck. As it enters the thorax it descends caudally between the two lungs and posteriorly of the left atrium of the heart in the mediastinum. It exits the thorax through its own oesophageal opening in the diaphragm

at the vertebral level T10. Once in the abdomen, the oesophagus is located directly posterior of the left lobe of the liver before turning sharply left connecting to the stomach through the cardiac orifice. The lower oesophageal sphincter is located at the entrance to the stomach. This sphincter plays an important role in preventing reflux of stomach contents and/or bile into the lower oesophagus (1).

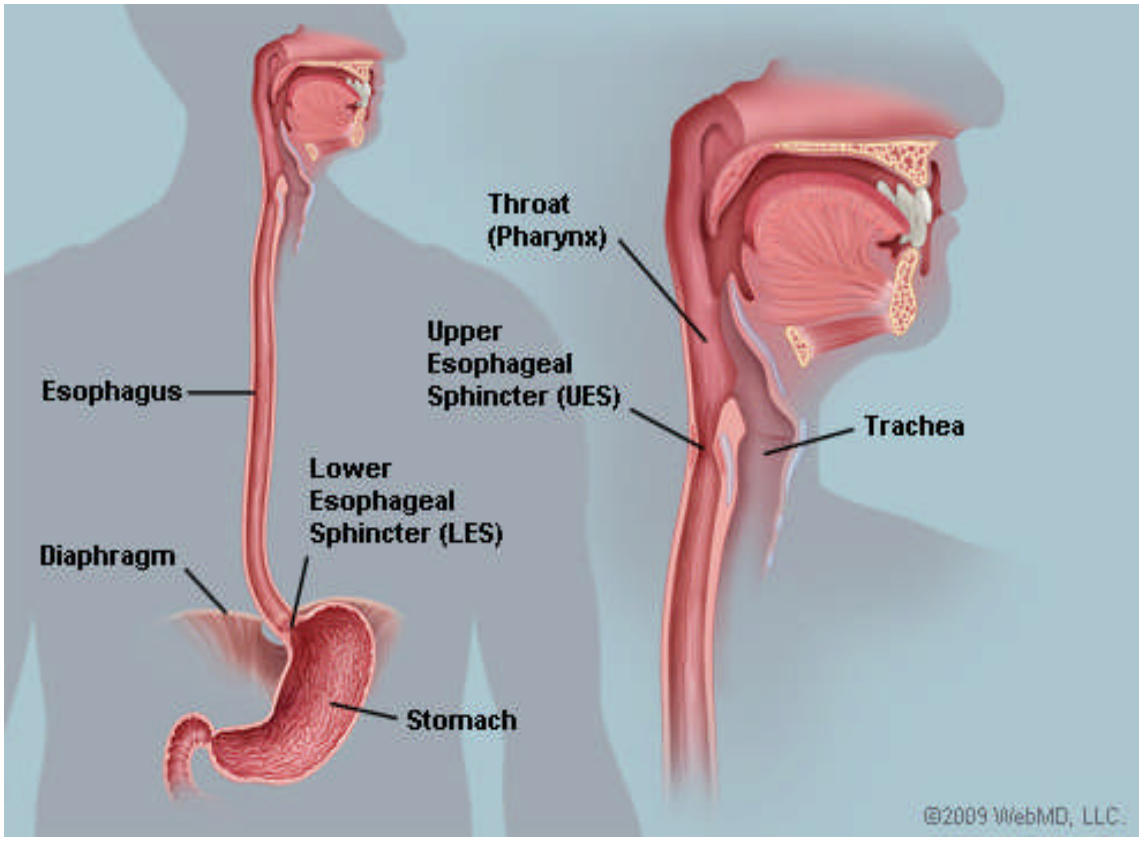


Figure 1: The upper gastrointestinal tract anatomy.
The diagram above illustrates the anatomy of the oesophagus and its relation to both, the oral and gastric cavity. Picture from www.webMD.co.uk (2).

The average length of the human oesophagus is 23-25cm with an average diameter of 1.5-2cm. It has a wall which consists of four layers (Figure 2);

a.) **Mucosa**: the inner most luminal layer consisting of epithelial cells and lamina propria which line the entire oesophagus. Most of the oesophagus is lined by non-keratinizing stratified squamous epithelium, only the intra-abdominal part is

lined by columnar epithelium. The location of the change in the epithelial phenotype is an important landmark in the oesophagus and is described as the Z-line. This line can be seen on endoscopic examination of the oesophagus.

b.) **Submucosa**: separated from the mucosa by the muscularis mucosae, the submucosa layer contains the oesophageal glands. These glands produce mucus to aid lubrication of food boluses on their passage towards the stomach.

c.) **Muscularis propria**: the muscularis propria consists of an inner circular and an outer longitudinal muscle layer. In the upper third, the outer layer consists of striated muscle and in the lower two thirds consists of smooth muscle responsible for peristalsis to move contents downwards toward the gastro-oesophageal sphincter.

d.) **Adventitia**: The outer most layer of the oesophagus consists of a thin layer of loose fibrofatty tissue which is mostly not covered by serosa with the exception of focal coverage by the pleura.

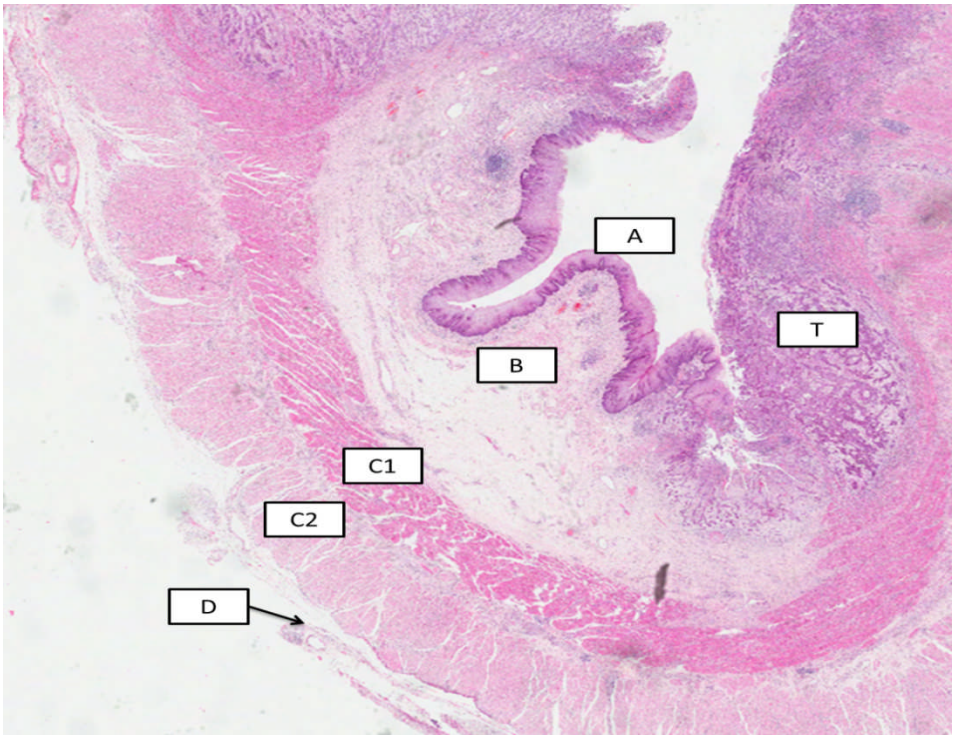


Figure 2: Haematoxylin & Eosin stained slide illustrating the layers of the oesophageal wall.
The diagram shows a microscopic view of the layers of the oesophagus. A= mucosa (squamous epithelium), B= submucosa containing glandular tissue, C1= circular muscle of muscular layer, C2=longitudinal muscle of the muscular layer and D= adventitia. T= malignant tumour

1.2 Histological subtypes

There are two main histological subtypes of oesophageal cancer (OeC): squamous cell carcinoma and adenocarcinoma. Squamous cell carcinomas originate from the non-keratinizing stratified squamous cells lining the oesophagus therefore are usually localised in the upper two thirds of the oesophagus. Adenocarcinomas are thought to originate from columnar (glandular) cells and typically occur in the lower third of the oesophagus. As well as histological and location differences, there is evidence to suggest that the pathogenesis of these two histological types of OeC is different (3). Squamous cell cancers usually develop in a stepwise process from mild to severe dysplasia and subsequently into an invasive squamous cell carcinoma (4). Adenocarcinoma of the oesophagus is thought to develop as a result of a sequence of

genetic and phenotype alterations known as the ‘metaplasia-dysplasia-neoplasia sequence’ (3, 5). This sequence begins with the normal lower oesophageal epithelial being persistently injured resulting in replacement of normal stratified squamous epithelium cells by abnormal columnar epithelial cells, also known as Barrett’s mucosa (6). It is thought that in the presence of continuous stimulation, this premalignant mucosa is prone to malignant transformation.

Other histological types of OeC are much less common and are therefore not featured in detail in this thesis.

1.3 Epidemiology of oesophageal cancer

1.3.1 Worldwide

Oesophageal cancer (OeC) is the eighth most common cancer in the world with 456,000 new cases diagnosed worldwide in 2012 accounting for 3.2% of all cancers diagnosed worldwide (7). OeC has been shown to cause 400,000 deaths worldwide in 2012 (7). There is a 2-4 times higher incidence in men than women and the highest incidence occurs in the 50-70 year old age group (8). The 2012 global cancer report indicated there were 281,000 OeC deaths in males compared to 119,000 in females (9). The overall incidence of OeC in Europe between 2000-2004 was 5.39 per 100,000 population in males and 1.13 per 100,000 for females, taken from the recent European cancer registry (EUROCARE) publication (10). The incidence of OeC shows substantial geographical variation globally. 80% of all reported cases are from areas found in the developing world (11, 12). The incidence rates worldwide range from 28.2 (Malawi) to 0.3 (Nigeria) age standardised rates per 100,000 in males and 20.8 (Malawi) to 0.3 (Nigeria) age standardised rates per 100,000 in females (7). The cumulative risk of developing OeC can vary 60 fold between countries worldwide and also between countries in the similar geographically areas (12). For example, there is a

twofold difference in developing oesophageal adenocarcinoma in Scotland as compared to the Republic of Ireland (0.60% risk vs. 0.29%, p<0.001) (12). Similarly, the overall incidence of OeC varies substantially between Southern African and West African regions. The age-standardised incidence rates (ASR) for men from Southern African areas is 22.3 cases per 100,000 population and 11.7 cases per 100,000 in women, compare this to the equivalent incidence for men in Western Africa where there is 1.4 cases per 100,000 population with 1 case per 100,000 in women (13). This dramatic variation in incidence has been of great interest to researchers over the past 20 years, leading to studies into genetic, environmental and culture differences between regions with contrasting incidences throughout the world (14-17).

1.3.2 United Kingdom

Oesophageal cancer (OeC) overall was diagnosed in 8,332 people in the United Kingdom (UK) in 2011 (18). Overall ASR of OeC in the UK is 6.4 per 100,000 populations, which is the highest in Europe (13, 19). It is the seventh most common cancer in males (ASR of 14.1 per 100,000) and in females, it is the fourteenth most common cancer in the UK (ASR of 5.3 per 100,000) (19). There has been an overall rise in incidence of OeC in the UK over the past 30 years, particular in males. The ASR of OeC has nearly doubled for males in the UK from 8.8 per 100,000 populations in 1975 (19). This rise is less apparent in females suggesting that the overall trend of rising OeC incidence cannot be explained by improved classification or diagnostic techniques (20, 21). However, a recent study suggests that the incidence of OeC in the UK has started to stabilise since 2002 (19).

1.3.3 Epidemiological differences between two main histological subtypes of oesophageal cancer

The frequency of the histological subtypes of OeC shows vast geographically variation across countries in the same continent, as well as regions of the same country (13, 22).

Global statistics show that squamous cell carcinoma makes up 87% of all oesophageal cancers diagnosed worldwide (398,000 cases), with a predominance in males (2:1 male: female ratio) (7). Adenocarcinomas accounted for 52,000 new OeC cases worldwide in 2012 with a male to female ratio of 4:1 (7). The incidence of squamous cell carcinoma by far exceeds that of adenocarcinoma in 90% of countries worldwide; however in some countries such as the UK and The Netherlands, adenocarcinomas are the predominant histological subtype of OeC (13, 19). Other countries showing this opposite trend compared to global figures are the United States of America (USA), Canada, New Zealand, Australia, Iceland, Sweden, Norway, Ireland, Malta and Cyprus (13, 23).

The highest risk areas for squamous cell carcinomas are found in the 'Asian oesophageal cancer belt' which includes Turkey, Iran, Mongolia and China among others. China itself, is responsible for more than half of the global squamous cell cancer cases, and squamous cell cancer of the oesophagus accounts for 23% of all cancer mortality in China (24). In Linxian, a rural community in the Henan province of northern China, there are more than 100 squamous cell carcinoma cases per 100,000 population per year, which is the highest reported prevalence of OeC in the China (11). However, outside this so called "cancer belt" zone, there are isolated areas worldwide which also report high incidences of squamous cell carcinoma of the oesophagus. South Africa has a significantly higher incidence of squamous cell cancer compared to the rest of Africa with incidence rates exceeding 100 cases per 100,000 population (17). Likewise there is also an isolated high incidence rate of squamous cell carcinoma (26 cases per 100,000 population) in northwest France in the Burgundy and Normandy regions (25). This high incidence is in contrast to the rest of northern Europe where the incidence rate is less than 4 cases per 100,000 populations. These findings suggest that there are likely environmental factors involved in squamous cell carcinoma

development. One study linked these isolated high incidences found in northern France to a regional culture of drinking hot alcoholic beverages (26).

There are also ethnic variations within countries such as in the USA where black Afro-American's have a 5-fold increased risk of developing squamous cell cancer compared to Caucasian Americans (27). Despite these high incidences in specific regions worldwide, the overall incidence of squamous cell carcinoma seems to have decreased or at least stabilised throughout the developed world in the past 30 years (24, 28, 29).

In the mid 1970's, adenocarcinomas of the oesophagus accounted for only around 5% of all cases of OeC worldwide (21). However, in contrast to squamous cell cancer, the incidence of adenocarcinoma of the oesophagus has increased over the past 30 years (30). Population-based studies in the USA and Europe indicate that the incidence of oesophageal adenocarcinoma has doubled between the 1970s and the late 1980s (31, 32). From there the incidence continued to increase by 5% every year (31, 32). Another study showed that the annual rise in the incidence of adenocarcinoma of the oesophagus in males was greater than that of any other cancer in the USA during the 1980s (33). This trend has also the been seen in the UK over the past 40 years where the adenocarcinoma incidence has risen sharply and to the point where adenocarcinoma of the oesophagus is now more prevalent than squamous cell carcinoma (19). In the UK, the 2012 overall age-standardised incidence of oesophageal adenocarcinoma was 7.2 per 100,000 in men and 2.5 per 100,000 in women which is the highest incidence of adenocarcinoma of the oesophagus in the world (7, 19, 21, 34).

1.4 Risk factors for the development of oesophageal carcinoma

1.4.1 Alcohol and tobacco

Tobacco (either smoked or chewed) and alcohol indigestion have been suggested to be the two main risk factors for OeC development (13). For squamous cell carcinoma in low risk, developed regions such as the USA and northern Europe, tobacco exposure and alcohol consumption are thought to account for up to 90% of cases (35). For both risk factors, there is some evidence that the quantity and duration of exposure are related to the risk of developing either subtype of OeC (36-40). There is however some evidence suggesting that smoking of tobacco (or ingestion) alone is an important risk factor for developing either subtype of OeC (38, 41-47). However, the exact mechanisms are still uncertain. Although both tobacco smoking and alcohol consumption appear to be independent risk factors, it has been suggested that in combination they may have a synergistic affect placing heavy smokers and excessive alcohol drinkers at the highest risk of developing OeC (27, 38, 48, 49).

1.4.2 Barrett's oesophagus and gastro-oesophageal reflux disease (GORD)

Barrett's mucosa results from the replacement of the normal stratified squamous cell epithelium by metaplastic columnar epithelium and was named after a surgeon called Norman Barrett who described this abnormal cell findings in 1950 (50). The mucosal change can be seen endoscopically and is usually confirmed by histological evaluation of tissue biopsies. Barrett's mucosa is associated with the development of adenocarcinoma of the oesophagus and is thought to be the most important precursor lesion (51-55). Barrett's mucosa results from recurrent cell irritation secondary to gastro-oesophageal reflux (GORD) where an incompetent lower oesophageal sphincter leads to acidic contents refluxing into the oesophagus (56). Therefore GORD has also been well described as a significant risk factor for adenocarcinoma of the oesophagus

through its role in the development of Barrett's mucosa (57-59). Recurrent acid exposure and related cellular irritation due to GORD is thought to trigger the metaplastic change from normal squamous epithelial cells to metaplastic columnar cells. However, only 5-8% of patients with GORD will develop Barrett's (60). Continuing stimulus (i.e. acid reflux) can induce dysplastic epithelial changes in some patients and eventually lead to invasive cancer (52, 55). Studies have shown that the length of the Barrett's mucosa is related to the severity of acid exposure (61, 62). However, there is still an ongoing debate as to whether the length of the Barrett's segments is associated with an increased risk of developing adenocarcinoma (62-64).

The estimated risk of developing adenocarcinoma from Barrett's mucosa varies across the published literature. A recent review of European available literature showed a range of 0.43 to 4.0% risk of progression from Barrett's mucosa to adenocarcinoma per year (65). The lowest risk (0.43%) was reported in a recent Dutch prospective study which was the largest carried out in Europe to date (n=42207) (66). However, other studies have shown the relative risk of developing adenocarcinoma in patients with Barrett's oesophagus is in the order of 50 to 100 times higher than someone without Barrett's mucosa (3, 60, 67).

1.4.3 Nutritional status/obesity

A person's nutritional status has been associated with a risk of oesophageal cancer (3). Malnourishment in the developing world or in low socioeconomic populations has been associated with low fruit intake and vitamin deficiencies, both of which have been shown to increase risk of developing oesophageal cancer (27, 68, 69). On the other hand, obesity has also been linked to a significant increased risk for developing adenocarcinoma of the oesophagus most likely as obesity is a major risk factor for GORD (30, 70, 71). The latter association may explain the rise in adenocarcinoma

incidence in countries such as the UK and the USA where there has also been a rise in the average body mass index (72-74).

1.4.4 Human papilloma virus (HPV)

Human Papilloma Virus (HPV) was first found to be associated with squamous cell carcinoma of the oesophagus more than 30 years ago when a study found that 40% (24/60) of OeC patients in the study had underlying HPV infection (75). In high risk areas for OeC such as China, eastern Asia, India and South Africa, HPV infection is known to be highly prevalent (76-78). Similarly, countries in Europe with regionally high rates of squamous cell OeC, such as France, also seems to have a high prevalence of HPV infection (79). However, despite this geographical relationship, causality between HPV infection and oesophageal squamous cell carcinoma pathogenesis has not been demonstrated so far.

1.4.5 Nitrosamines

Another associated risk factor for OeC is exposure to nitrosamines which are found in high levels in preserved fish and salted or pickled vegetables, all of which are popular in high prevalence areas such as Linxian in northern China. Furthermore, recent evidence suggests that chewing betel nuts (popular in China and other high prevalence regions), which is high in nitrosamines precursors, increases the risk of developing squamous cell OeC by up to 13 fold (16, 80, 81). This risk is further increased in patients who also smoke or drink alcohol as well as chewing betel nuts, with a recent large study finding a 9-20 fold increased risk if two of the risk factors where present and a 41 fold increased risk if a patient used all three substances (38).

1.5 Oesophageal cancer treatment options

The majority of UK patients present with advanced disease which is often not locally resectable or with distant metastatic disease (82). In this situation, patients are

managed with palliative intent which can involve chemotherapy, radiotherapy or endoscopic measures. Overall 5 year relative survival in these patients can be as low as 3.1% (83). However this thesis will focus on OeC when patients present with potentially resectable disease.

1.5.1 Surgical treatment options

Surgical resection of OeC remains the only curative option despite advances in medical chemotherapy, radiotherapy and endoscopic interventions. Potential curative surgery is considered for OeC patients presenting with locally advanced disease i.e. Tumour Node Metastasis (TNM) classed stage II or III disease (84, 85). In the UK, only around 35% of patients present with potentially curable disease (82).

Surgery involves the resection of the oesophagus including the tumour mass, surrounding lymph nodes, lymphatics and any resectable surrounding structures that are thought to have been infiltrated by the cancer. Surgical approaches commonly used are the transhiatal or transthoracic approach. In the UK, a transthoracic approach is commonly used such as an Ivor Lewis oesophagectomy (abdominal and right thoracic approach) or the three-incision modified McKeown oesophagectomy that involves laparotomy, right thoracotomy, and neck anastomosis (86-88). The choice of surgical approach depends on the location of the tumour and the preference of the surgeon (89). Over the past 10 years minimal invasive oesophagectomy has become more commonly used in the UK which involves the use of laparoscopy to perform part or sometimes all of the same procedure. Initial studies identified reduced median stays in intensive care, with initial decreased morbidity and mortality following minimally invasive surgery (90-92). However there remains uncertainty with regards to whether minimal invasive surgical techniques are superior to open surgery in terms of overall outcomes and complication rates hence there is currently a large randomised control

trial ongoing to address this debate (93).Currently following surgical management the 5 year survival in the UK has been reported as 23% (94).

Over the past 10 years the treatment of patients with locally advanced resectable OeC has changed following large multi-centre trials showing that neoadjuvant chemo(radio)therapy improves patient survival (95-97). This is despite the fact that in around 50% of the patients, no radiological measurable local response was achieved (98). The first trial to highlight the possible benefit of neoadjuvant chemotherapy in OeC was the UK medical research council (MRC) OE02 trial (99) (see 3.1 Background-OE02 MRC trial). Figure 3 shows a typical treatment algorithm used to differentiate cases and guide management of individual oesophageal cancer patients (85). There are two decision points in the patient pathway: (1) based on clinical staging (determined from imaging via Computerised Tomography scans (CT), Positron Emission Tomography (PET) or endoscopic ultrasound scan (EUS) and direct endoscopic examination) and (2) pathological tumour staging after surgical resection.

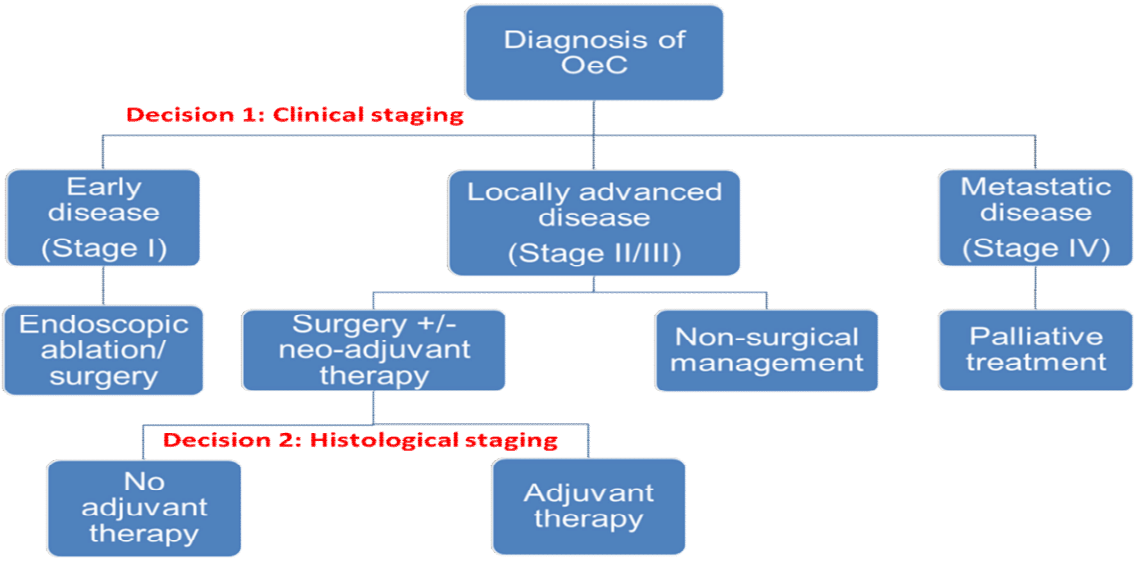


Figure 3: UK oesophageal cancer patient treatment algorithm.
The flow chart above illustrates how disease stage influences the management of OeC patients in the UK. The first treatment decision point is based on clinical staging where one needs to differentiate between early, locally advanced and metastatic disease. The second treatment decision point is in patients who have undergone surgery and is dependent on the pathological stage based on the resected specimen.

1.5.2 The role of neoadjuvant chemotherapy in the management of patients with oesophageal cancer

In the 1980s, 5 year survival of patients with OeC was <10% in the USA despite radical resection (100, 101). This prompted studies looking at improving survival rates by using pre-operative (neoadjuvant) chemotherapeutic agents, such as Vindesine (102-104) and Bleomycin (105, 106) as well as Cisplatin (107, 108). A study by Kelsen et al showed that the use of pre-operative chemotherapy in patients with squamous cell carcinoma induced tumour regression and resulted in higher resection rates (102, 109-111). Two large multi-centre randomised control trials were conducted in the early 1990's (99, 112). The first one was conducted by the US Radiation Therapy Oncology Group (RTOG) lead by Kelsen (112) involving 440 patients who were randomly allocated to either surgery alone (n=227) or chemotherapy followed by surgery (n=213). Patients in the chemotherapy followed by surgery group received three cycles of Cisplatin and 5-Fluorouracil. The trial recruited patients with squamous cell

carcinoma (46%) and adenocarcinoma (54%) with locally advanced, resectable disease. Patients with distant metastasis (M1 disease) were excluded. Responders and patients with an R0 (complete resection with no involved margins) also received two further cycles of chemotherapy post operatively. Patients were recruited over 5 years from 123 institutions across the USA (1990-1995). This study found no significant difference in overall and disease free survival between the two groups (112). It was speculated that earlier studies in smaller patient cohorts may have overestimated the potential effect of neoadjuvant chemotherapy (112). Subsequent randomised controlled trials (113, 114) in the USA using chemotherapy alongside radiotherapy preoperatively were more successful and established pre-operative chemo(radio)therapy as the standard of care in the US for patients with locally advanced potentially curable OeC (115).

The other major randomised control trial was the OE02 trial carried out by the UK MRC (99). The OE02 trial was a multi-centre randomised trial which compared patients undergoing neoadjuvant 5-FU/cisplatin chemotherapy followed by surgery with patients treated by surgery only. It included patients from the UK and The Netherlands. This trial is discussed in more detail later (3.1 Background- OE02 MRC trial). In summary, the results showed a significant improvement in overall and disease free survival in patients who had neoadjuvant chemotherapy ($p=0.04$ and $p=0.0014$, respectively) compared to those who underwent surgery alone changing the clinical practice in the UK (99). In the UK following the OE02 trial neoadjuvant chemotherapy followed by surgical resection has become the ‘gold-standard’ treatment option for locally advanced (stage II or III) oesophageal cancer.

The most recent meta-analysis by Sjoquist et al (n=4188) looked at literature comparing surgery alone with neoadjuvant chemotherapy or neoadjuvant chemoradiotherapy followed by surgery in patients with resectable OeC (116).

Although no significant difference in survival was seen between patients who had neoadjuvant chemotherapy compared to those who had neoadjuvant chemoradiotherapy followed by surgery, a clear difference in survival was seen between patients who received some form of neoadjuvant treatment followed by surgery to those who were treated by surgery alone (p=0.005; hazard ratio 0.87; 95% confidence interval 0.79-0.96) with an overall 2 year survival benefit of 5.1% (p=0.005) (116).

1.5.3 Challenges of neoadjuvant chemotherapy

The aim of neoadjuvant chemotherapy is to downstage the primary tumour to increase the chance of a complete resection of the primary tumour and to prevent micro-metastasis to lymph nodes or distant sites, therefore reducing tumour recurrence. Managing a patient who receives or has received neoadjuvant chemotherapy can be challenging as toxicity complications can lead to increased morbidity and mortality (117). Chemotherapy may have side-effects ranging from hair loss and gastrointestinal effects to acute chemotherapy toxicity which can lead to the patient being unable to complete their regimen or can even be life threatening (118). Figures from the most recent UK National Oesophagogastric Cancer Audit (NOGCA) suggest that nearly 3 in 10 patients are unable to complete their neoadjuvant chemotherapy course, mainly due to the development of acute chemotherapy toxicity (82). Furthermore, chemotherapy does not always lead to the intended preoperative disease control/tumour regression and in fact disease progression may occur during this neoadjuvant treatment stage. The NOGCA figures suggest that 4.5% of patient had disease progression during their pre-operative chemotherapy (82). Lower rates were seen in the OE02 trial (1.4%) (99) but higher rates in the RTOG randomised control trial (6.9%) (112). Unfortunately, there is no clinically established biomarker to predict which patient might benefit from

neoadjuvant chemotherapy and which patient might be at risk of severe chemotherapy related side effects.

1.6 Oesophageal cancer patients and survival

Overall 5 year survival of all patients with OeC irrespective of disease stage is reported between 9-15% (19, 119, 120). Figures from EUROCARE show an overall 1 year survival rate of 35-37% and 5 year survival rates of 10-13% (10). In England, overall survival rates are slightly lower than the European average, with 1 year survival at 31-35% and 5 year survival 8-10%, respectively (10).

A recent study described a worldwide 5 year overall survival for patients with stage I (early disease) oesophageal cancer disease of 60-80% (121). Worldwide figures for 5 year survival of patients who have locally advanced disease, and then undergo surgery (typically stage II-III disease) ranges from 22-37% (83, 122-125). This relatively poor prognosis is despite improvements in detection techniques, better surgical equipment, advanced medical oncology treatment options and despite increasing public disease awareness.

Recent studies suggest patients with squamous cell OeC have a worse prognosis compare to those with adenocarcinoma OeC (126-130). This may be related to the fact that patients with squamous cell OeC tend to have more significant co-morbidities, older and have therefore have higher adjusted overall post-operative death risk (126, 131). Another potentially important factor is that 75% of squamous cell OeC are found to be in contact with the trachea making surgery more difficult increasing post-operative complications such as a tracheal fistula as well as increasing the risk of the tumours being unresectable (126).

1.7 Tumour node metastasis classification for oesophageal cancer

The Tumour Node Metastasis (TNM) classification is currently the only established prognosis prediction tool and is used for most malignant tumours worldwide. The TNM classification was introduced in the 1940s by Pierre Denoix using different parameters such as tumour size, depth of invasion of the primary tumour, lymphatic involvement, and presence of metastases to classify cancer (132, 133). The initial work from Denoix lead to the formation of the International Union Cancer Committee (UICC)(134) and the American Joint Committee on Cancer (AJCC) leading to the introduction of a formal cancer staging system (135, 136). Nowadays, the TNM classification is used throughout the world to clinically predict patients' prognosis as well as assist clinicians to determine patient treatment (137).

In 1973 the first recognised staging system specifically aimed at classifying OeC was introduced and was quickly followed by the second edition in 1977. Both these editions only involved a clinical classification of T category by tumour length, position and the presence of obstructive symptoms. In the 3rd edition of TNM both pathological and clinical classification of the T category was introduced but as two separate categories (T and pT staging). Following publications from Japan the 4th edition of the TNM classification in 1985 focussed on depth of tumour to determine the T category (138). Following on from similar subsequent editions of the TNM classification of OeC, there have been significant changes in the most recent 7th edition most notably in the classification of nodal status (N category), which now has 4 categories depending on the actual number of positive lymph nodes (139). This is major change from 2 categories in previous editions based on whether there was at least one positive node (140). Although these changes in staging are aimed to reflect changes in evidence of

best clinical practice, there has often been controversy and debate. The most recent 7th TNM edition (139) implemented changes for the staging of OeC (

Table 1) based on results from evidence taken from the worldwide oesophageal cancer collaborative (WECC) (141).

Table 1: UICC TNM classification (7th ed.) of oesophageal cancer (139)

Primary tumour (T category)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	High grade dysplasia
T1a	Tumour invades lamina propria or muscularis mucosae
T1b	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4	Tumour invades surrounding structures
T4a	Tumour invades pleura, pericardium or diaphragm
T4b	Tumour invades other structures such as vertebrae or aorta
Regional lymph nodes (N category)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis involving 1-2 nodes
N2	Regional lymph node metastasis involving 3-6 nodes
N3	Regional lymph node metastasis involving 7 or more nodes
Distant metastasis (M category)	
MX	Distant metastasis cannot be assessed
M0	No evidence of distant metastasis
M1	Distant metastasis present

Only data from patients who had been treated by only surgery alone was used to establish the revised TNM classifier (Table 1). Thus, the accuracy and validity of the TNM classification for patients treated with neoadjuvant chemo(radio)therapy is currently still uncertain (142-148). With the apparent change in clinical practice to treat

oesophageal cancer patients with neoadjuvant or peri-operative chemo(radio)therapy, there is a need to establish the prognostic value of the TNM classification in this setting and investigate whether there are other prognostic factors.

1.8 Radiological prognostic markers in patients with oesophageal cancer

Radiological imaging plays an important role in establishing the clinical stage of cancer influencing patient management (Figure 3). Endoscopic ultrasound (EUS) allows visualisation of the lesion and assessment of the depth of invasion (T category). The use of EUS has been shown to be superior to Computed Tomography (CT) or Positron Emission Tomography (PET) imaging in assessing locally advanced disease for the determination of depth of invasion of the primary tumour and lymph node status (149-151). However, EUS imaging is limited to a depth of 5cm and thus cannot be used to assess distant disease so is usually used in conjunction with other imaging modalities (150, 152). CT images are relevant for the clinical staging of OeC to localise the tumour and assess any potential involvement of surrounding structures and/or lymph nodes. CT scans also enable the identification of any distant metastases and are the imaging of choice for the detection of local or distant recurrence during the post-operative follow up. With respect to the best modality of staging OeC, the current literature is still controversial (153-156).However a recent meta-analyses suggested that PET may have a higher diagnostic performance in detecting distant metastasis (157, 158).

PET imaging is based on the principle that malignant cells can have an increased uptake of glucose (159). Fluorine-18 fluorodeoxyglucose (FDG) is used to identify higher uptake in malignant cells in comparison to the uptake of the normal tissues. The use of PET imaging for staging OeC patients is relatively new and its value in

identifying tumour regression after neoadjuvant therapy remains to be established (160, 161).

PET scans may have a potential advantage over conventional CT scans in the detection of lymph node metastases. Radiologists currently use size and shape criteria to determine whether a lymph node maybe involved with cancerous cells or not when assessing CT images (157). CT scanning has a poor sensitivity for correct lymph node staging (162, 163). However, as PET scans detect metabolic changes in the lymph node tissue which can be related to the infiltration of the lymph node by tumour, PET has been shown to have a higher sensitivity to detect distant lymph node involvement than conventional CT scans (164). A meta-analysis showed the sensitivity of detecting distant lymph node involvement was 71% in PET imaging compared to 52% using CT imaging (157). However, when involved lymph nodes are in close proximity to the primary tumour, the FDG uptake by the primary tumour may be indistinguishable from that of surrounding lymph nodes. It has been demonstrated that FDG PET had 33% sensitivity to detect lymph node metastasis close to the primary tumour as opposed to 81% sensitivity of EUS imaging (165).

Recent studies have shown the overall accuracy in TNM staging improved from 83-86% for PET imaging alone to 90-92% when PET imaging is done in conjunction with CT (166, 167).

1.9 Tumour regression systems used to assess oesophageal cancer

Patients with potentially curable OeC, who show response to neoadjuvant treatment seem to have significantly improved survival than those who do not respond, especially if they have a complete pathological response e.g. no viable tumour in the resection

specimen (111, 168-173). Pathologic examination of the resection specimen after neoadjuvant chemotherapy remains the “gold standard” for evaluation of tumour response (98, 174). However, there I no consensus regarding which histopathological tumour regression system should be used for oesophageal cancer resection specimens (121, 175, 176).

A number of different tumour regression assessment systems have been suggested for oesophageal cancer treated with neoadjuvant chemotherapy (175-180). The table below (Table 2) summarises the details of some of the published scoring systems which have all shown a relationship with OeC patient prognosis following neoadjuvant therapy. There have also been recent attempts to incorporate regression grading of tumour involved lymph nodes along with at the primary site to try to create a more comprehensive scoring systems (181-183), however, grading of regression in lymph node metastases will not be assessed in this thesis.

Table 2: Published tumour regression scoring systems for oesophageal cancer

Authors	Grading	Pathological features
Mandard et al 1994 (177)		
	TRG1	Complete regression (i.e. fibrosis without detectable residual cancer cells)
	TRG2	Few residual cancer cells scattered through the fibrosis
	TRG3	Fibrosis and tumour cells with predominance of fibrosis
	TRG4	Residual cancer outgrowing fibrosis
	TRG5	Absence of any regressive changes
Japanese Society of Esophageal Disease (184)		
	ypV0	Ineffective (i.e. no regression evidence)
	ypV1	Slightly effective: Viable cell more than 1/3 of tumour tissue, but with evidence of degeneration
	ypV2	Moderately effective: Viable cell less than 1/3 of tumour tissue and severely degenerated or necrotic
	ypV3	Markedly effective: No viable cell
Schneider et al 2005 (185)		
	I	>50% vital residual tumour cells
	II	10%–50% vital residual tumour cells
	III	<10% vital residual tumour cells
	IV	no vital residual tumour cells
Chirieac et al 2005 (179)		
	1	No evidence of residual tumour
	2	1-10% residual tumour
	3	11-50% residual tumour
	4	>50% residual tumour
***Becker et al 2003 (178) Swisher et al 2005 (186) Langer et al (175)**		
	CRT	no residual cell
	P1	1%–50% of residual viable cell
	P2	>50% residual viable cell in primary tumour
Brucher et al 2006 (176) Barbour et al 2008 (187)		
	Responders	<10% residual tumour cells
	Non responders	>10% residual tumour cells
Donington et al 2003 (188)		
	Complete responders	No evidence of residual tumour
	Residual tumour	Any evidence of residual tumour

*Becker et al original paper developed the system originally for gastric cancer and later used the same system for oesophageal cancer

1.9.1 Tumour regression grading according to Mandard et al – the original system

A classification system to assess tumour regression in OeC after radiotherapy was first suggested by Mandard et al (177). This initial study, evaluating specimens from 93

OeC patients with squamous cell cancers led to the definition of 5 grades based on the relative extent of fibrosis. Mandard et al suggested a significant difference ($p<0.001$) in disease free survival between patients with TRG 1-3 compared to patients with TRG 4-5 (Table 2). The Mandard TRG classification has been used in a number of OeC studies since to assess tumour response to neoadjuvant therapy (189-192). The Mandard TRG classification is currently used for the assessment of OeC resection specimens by many pathologists in the UK. There have been concerns that the determination of the TRG is very subjective and was particularly difficult to reproduce reliably, especially when differentiation between TRG 2, 3 and 4 is required (181). Studies have shown that observers often find it difficult to quantify fibrotic changes as distinguishing between fibrosis (e.g. tumour regression) and desmoplastic stroma (a normal component of the non-regressed tumour) is inherently difficult (174, 191-193).

1.9.2 Tumour regression grading according to Becker et al - the European perspective

Becker et al agreed with Mandard's findings that tumour regression leads to the formation of fibrosis and fibro-inflammatory areas (178), however, they suggested an alternative regression classification system which estimates the percentage of residual viable tumour cells in the whole of the tumour bed. Becker et al proposed a 4 tier classification system as opposed to Mandard's 5 categories (see Table 2). The Becker TRG classifier was initially tested in gastric cancer specimens and showed a relationship with patient survival on univariate analysis (178). The same authors summarised their original classification to a 3 or 2 tier system (175, 176).

1.9.3 Tumour regression grading according to Chirieac et al - the American perspective

Chirieac et al proposed a regression grading classification with initially 4 groups and later 3 groups (Figure 4). Their study showed a significant difference in disease free

survival using the 4 tier classification system (194) and subsequently with a 3 tier classification (186). Using the three tier classification, the percentage of residual tumour cells found in a resection specimen was significantly related to patient survival (3 year survival TRG1 (0% residual) =74% vs. TRG (1-50%) =54% vs. TRG3 (>50%) = 24%; p<0.001).

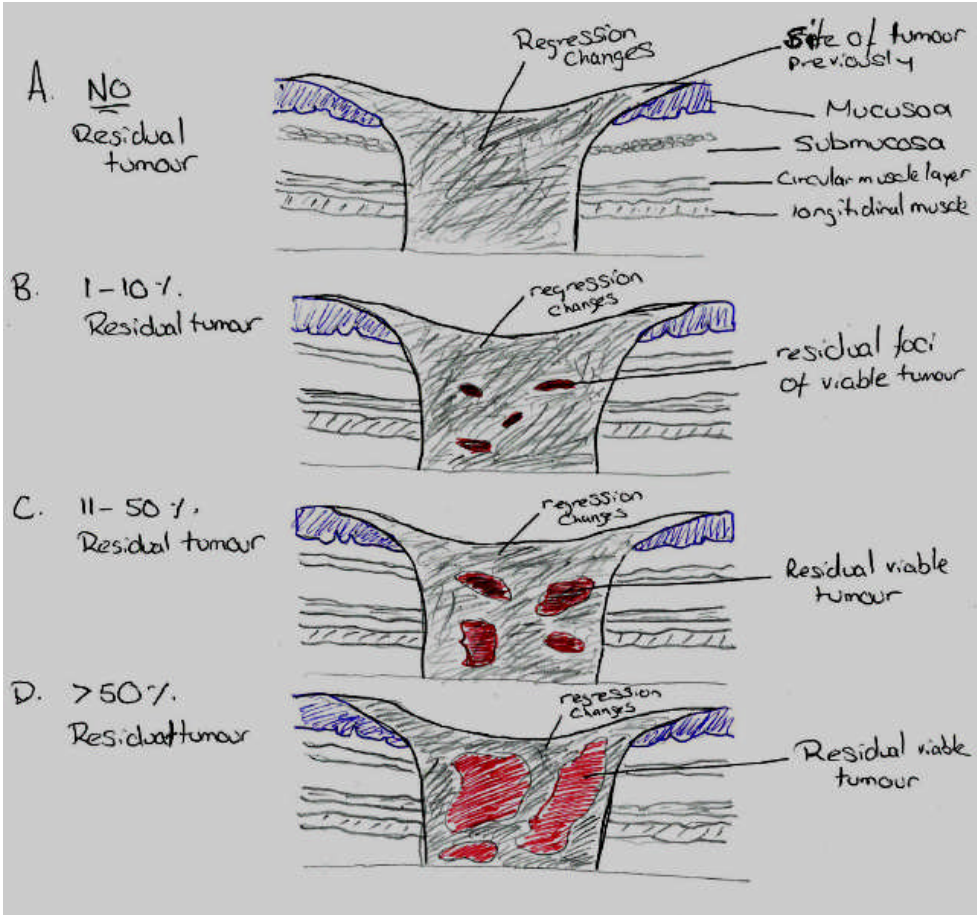


Figure 4: Chirieac et al 4 tier tumour regression grading system.
Illustration showing a cross-section picture of a primary oesophageal tumour after neoadjuvant chemotherapy. (A) No evidence of residual tumour within the tumour bed (area of inflammatory and fibrotic changes). (B) Between 1-10% of residual tumour cells, fibrosis and inflammatory changes predominate. (C) Between 11-50% of residual tumour present at site of primary tumour. (D) Greater than 50% residual tumour remaining. Diagram adapted from Chirieac *et al* (179).

1.9.4 Japanese tumour regression grading for oesophageal cancer

Since 1974, Japan has used its own regression scoring system established by the Japanese Society of Esophageal Disease (195). This current 4 tier system is based upon identification of the site of the original tumour ('tumour zone') on haematoxylin

and eosin stained slides. The percentage of tumour within the tumour zone is then calculated by dividing the sum of areas with viable tumour by the overall tumour zone and cases are classified as ypV0-ypV3 depending on the percentage (184). This tumour regression classification was found to be an independent prognostic marker for overall survival in patients with OeC treated by pre-operative chemoradiotherapy ($p<0.001$).

1.9.5 Challenges of the current tumour regression grading systems

One of the difficulties in establishing which TRG classification is 'best' is that different studies have used patient cohorts with different neoadjuvant treatment regimens (chemotherapy alone vs chemoradiation), different histological subtypes and applied different regression grading system. The majority of TRG studies to date have examined tumour regression after neoadjuvant chemoradiotherapy in OeC (174, 176, 177, 179, 180, 186, 189). The Japanese society of esophageal disease and the Mandard regression grading are being used the most worldwide and favour systems with 5 or 4 grading categories. Recent European and American studies are favouring classifications with fewer categories suggesting that such classifications will be easier to implement with less inter-observer variability (174, 176, 179).

The main criticism for all the TRG classifications has been high intra-observer and inter-observer variability with Kappa scores as low as 0.28 (174, 192, 196, 197). The high intra- and inter-observer variability could potentially be reduced by using a more objective quantitative method or an automated computer generated method. Recent work in colorectal cancer used a quantitative morphometric method called 'point counting' to establish a "tumour density score" per case (198, 199). The same methodology could be used in post-chemotherapy resection specimens and might improve the inter-observer agreement as predefined measurement points need to be

assessed which will provide a quantitative measure of the relative amount of tumour versus stroma/fibrosis.

Another challenge is that assessment of using any of the current TRG classification system requires embedding the whole of the tumour bed and can only be performed after surgery. It would be desirable as well as be of clinical interest to have a prognostic TRG classification that does not depend on embedding of the whole tumour bed and could be applied while the patient is still under pre-operative treatment e.g. could be assessed in endoscopic biopsies. The current thesis will address these challenges.

1.10 Circumferential resection margins in oesophageal cancer

Local OeC recurrence after attempted curative surgery often results in only palliative options being available for treatment. A R0 resection indicates that longitudinal and circumferential margins are clear of any macroscopic or microscopic tumour (200). While an R1 resection indicates evidence of microscopic residual tumour and R2 resection implies evidence of macroscopic tumour, therefore an incomplete resection. Depending on the pathological definition used, a positive (e.g. R1) resection margin can either mean that there is viable tumour directly at the margin (definition used by College of American Pathologists) or there are tumour cells with 1mm of the resection margin (definition used by the Royal College of Pathologists UK). There are currently conflicting results as to whether one of these definitions is better than the other with respect to predicting patient's prognosis (201-203). Multiple studies have shown the prognostic importance in achieving an R0 resection (84, 112, 204, 205). Recently, a meta-analysis using data from 30 European centres including 2060 patients identified the presence of tumour at the tumour margin to be an independent prognostic marker for survival ($p<0.001$) (205). However this included patients who had undergone a

variety of treatments including those who had surgery alone and patients who underwent various regimes of chemo(radio) neoadjuvant therapies.

The presence of a ‘positive’ circumferential resection margin has been associated with a poor prognosis in OeC patients who undergo surgery alone (201, 203, 204, 206, 207). However in cohorts including patients who have undergone neoadjuvant treatment there have been conflicting results with some showing circumferential resection margin remains a prognostic marker (208-212), while other studies have shown in this setting following neoadjuvant treatment the circumferential resection margin is not a prognostic marker (213-216).

1.11 Tumour infiltrating immune cells in oesophageal cancer

The immune reaction towards the tumour cells has been proposed as the seventh hallmark of cancer (217). There is growing evidence that the interaction between malignant cancer cells and the host’s immune cells has an important role in tumour progression (217-220). The concept of immunosurveillance and immunoediting of the primary malignancy has led to the use of immune targeted therapy (immunotherapy) being used in lymphoma (221, 222). Intratumoural immune cells are thought to have different functions with regards to being pro-tumour or anti-tumour in nature (218, 219, 223). Also different subsets of the same immune cell type, such as macrophages, have been shown to exhibit different effects on tumour cells (224, 225). There have been a number of studies recently which have identified the level of lymphocyte infiltration of the tumour as being related to patient prognosis (226-232), and identified macrophage infiltration of the tumour as a poor prognostic marker (233-241). Neutrophils have also been shown to have pro-tumour actions with high infiltration levels in the tumour being related to poor prognosis (235, 242-247).

In OeC, the evidence suggests that high tumour infiltration by lymphocytes is related to a good prognosis (248-255). However, studies (248-256) are difficult to compare as they used different neoadjuvant therapy regimes and various scoring methods. No study to date has used a technique to quantify the level of lymphocyte infiltration. Recent studies have shown that the ratio of “anti-tumour” lymphocytes to “pro-tumour” macrophages may be the most important prognostic marker with regards to immune cell distributions at the tumour microenvironment (237, 257).

AIMS OF THE STUDY

This thesis aims to identify new post-operative prognostic markers for patients with locally advanced resectable oesophageal cancer.

The specific aims are to

1. Investigate the prognostic value of the circumferential resection margin (CRM) involvement after pre-operative chemotherapy and establish whether the presence of tumour cells within 1mm has the same prognostic value as the presence of tumour cells directly at the margin.

2. Determine whether tumour cell density measurements at the luminal surface of the resection specimens can be used to reliably and reproducibly predict OeC patients' prognosis in patients treated by surgery alone as well as in patients treated with neoadjuvant chemotherapy.

3. Evaluate the prognostic value of the tumour infiltrating immune cells in OeC patients treated by surgery alone as well as patients treated with neoadjuvant chemotherapy and to try to establish whether immune cell ratios at the tumour microenvironment may also allow prognostic stratification.

HYPOTHESES

The hypotheses for this work are:

1. The presence of tumour cells within 1mm, as well as directly at the circumferential resection margin (CRM) is related to poor prognosis in oesophageal cancer patients who have undergone neoadjuvant chemotherapy followed by surgery.

2. The tumour cell density will vary between cases who have had neoadjuvant chemotherapy and those who have been treated by surgery alone. A high tumour cell density at the luminal surface is related to a poor prognosis in patients treated by neoadjuvant chemotherapy followed by surgery.

3. The distribution and proportion of different immune cells in the tumour microenvironment will vary between those who have undergone neoadjuvant chemotherapy followed by surgery and those who underwent surgery alone. Immune cell infiltration of the tumour is related to prognosis in oesophageal cancer patients.

2. THE PROGNOSTIC ROLE OF THE CIRCUMFERENTIAL RESECTION MARGIN STATUS IN PATIENTS WITH OESOPHAGEAL CANCER

Work presented in this chapter has previously been published and the following sentence is included in order to comply with copyright rules requested by the respective publishing journal:

This is a pre-copy-editing, author-produced PDF of an article accepted for publication in the European Journal of Cardiothoracic surgery (258) following peer review. The definitive publisher-authenticated version of "Prognostic significance of cancer within 1mm of the circumferential resection margin in oesophageal cancer patients following neo-adjuvant chemotherapy" (562-567.doi: 10.1093/ejcts/ezs331) is available online at: http://ejcts.oxfordjournals.org.

2.1 Background

Depending on the type of oesophagectomy performed, the resection of an oesophageal cancer involves mobilisation of the stomach plus in some cases of the duodenum, then resection of a segment of the oesophagus containing tumour, along with the tissue that surrounds it such as fat, lymph nodes and pleura. In the UK, an oesophagectomy is most commonly done as a two stage procedure known as an Ivor Lewis oesophago-gastrectomy. The oesophageal segment and the proximal stomach are removed before the remnant stomach is pulled up and an oesophageal to stomach anastomosis is created in the chest. The resected specimen is either sent intact or after lymph node dissection for pathological assessment.

Part of the dataset requirements published by the Royal College of Pathologists UK (RCPath), is the assessment of whether a complete resection of the tumour (e.g. an R0 resection) has been achieved (200). For an R0 resection both longitudinal (proximal

and distal) resections margins as well as the circumferential (sometimes also called radial) resection margin must be free of tumour. R1 would indicate that there are microscopically visible tumour cells present; R2 is defined as the presence of macroscopically visible tumour at the resection margin. Several large studies have demonstrated the importance of an R0 resection for patient survival following surgery for an oesophageal carcinoma (84, 112, 204, 205). There is evidence that involvement of either the proximal or distal resection margin increases the risk of local recurrence (94, 259). However, the prognostic importance consequence of an involved circumferential margin remains controversial (214, 260, 261).The circumferential resection margin (CRM) is usually assessed pathologically by slicing the fixed oesophagectomy specimen perpendicular to its longitudinal axis (Figure 5a & 5b).

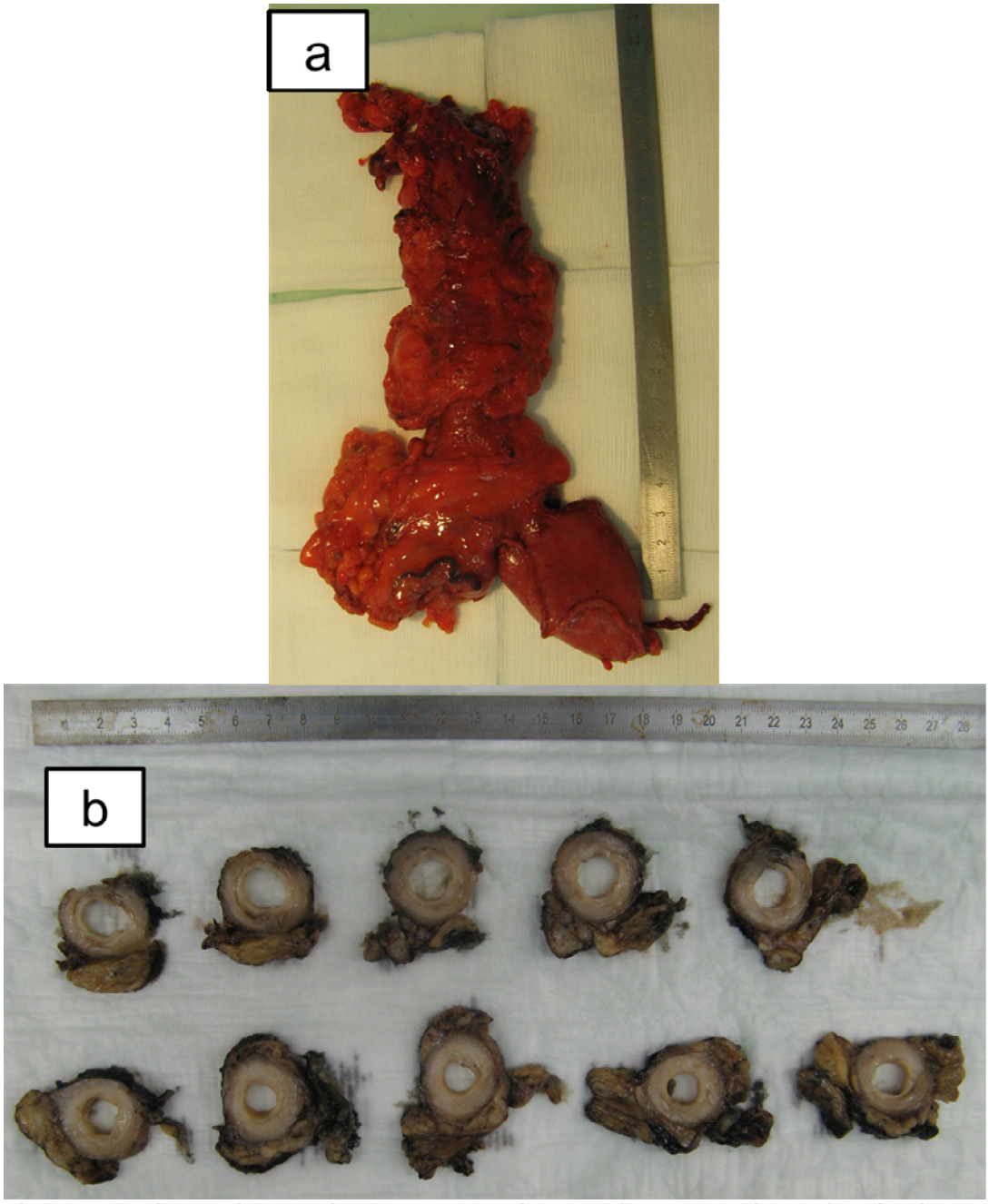


Figure 5a & 5b: Fresh oesophagectomy specimen and cross-sections after fixation. **(5a)** Photographs showing the fresh (unfixed) resection specimen as it arrives in the pathology department. **(5b)** photographs of cross-sections cut after fixation of the specimen. The oesophageal lumen and wall can be identified as well as surrounding tissue resected along with the oesophagus.

Figure 6 shows a diagrammatic representation of the assessment of the distance of clearance from the CRM, a surgically created boundary. A circumferential resection margin (CRM) is defined as 'positive' by the RCPath if viable tumour cells are found within 1mm of the resection margin (200). The College of American Pathologists (CAP)

defines a positive CRM as evidence of viable tumour cells directly at the resection margin (202).

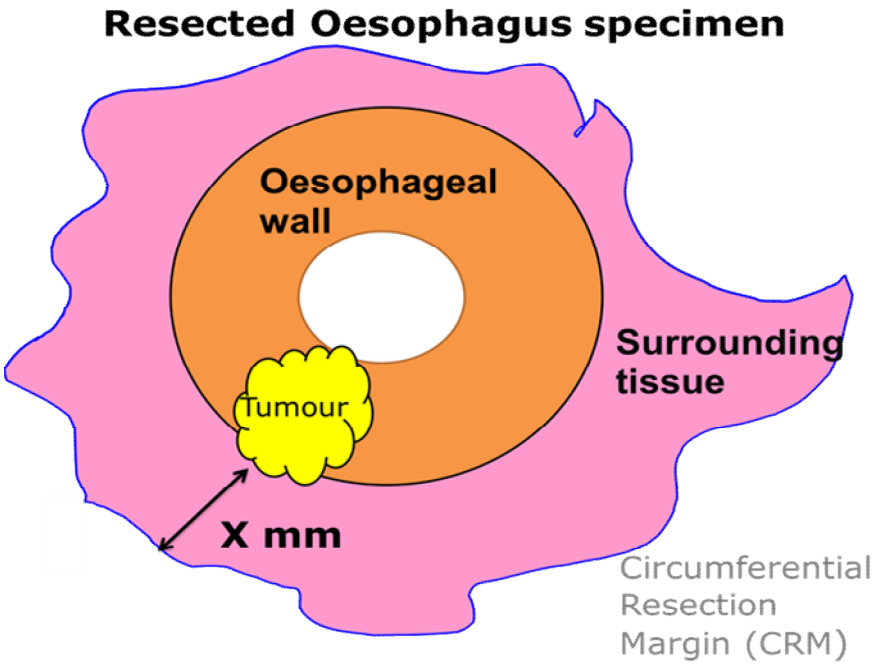


Figure 6: Cross-sectional view through an oesophagectomy specimen.
The circumferential resection margin is outlined in blue and represents the outermost margin of the specimen. Centrally located is the oesophageal lumen. The orange area represents the wall of the oesophagus including its layers the mucosa, submucosa and muscularis propria. Surrounding tissues (adventitia, fat) is represented in pink. Tumour area is represented in yellow. The CRM status is determined by measuring the minimal distance of viable tumour cells from the margin**.**

The prognostic significance of CRM involvement using the RCPath definition was first suggested by Sagar et al in a small series of OeC cancer patients treated by surgery alone. This initial and subsequent study by the same authors demonstrated that the presence of cancer cells within 1mm of the CRM (RCPath CRM-positive) was associated with a poorer prognosis (206, 207). They went on to identify a positive CRM to be an independent prognostic marker for overall survival and also associated with an increased risk of local recurrence (206). Further studies examining the relationship between CRM status and prognosis in patients with OeC are summarised in Table 3. CRM has been identified as a prognostic factor in oesophageal cancer patients treated

with surgery alone in multiple studies (201, 204, 206, 207, 210, 262). The studies assessing the prognostic value of the CRM status after neoadjuvant chemotherapy have often also included patients who also had surgery alone (211-213, 215, 216, 260, 263). At the time of this study, there was one study published which had examined a homogenous cohort of neoadjuvant chemotherapy followed by surgery patients (209) and one study where patients were treated by chemoradiotherapy followed by surgery (208). Both studies found that CRM status was a prognostic marker after neoadjuvant therapy. However, there are other studies with mixed treatment cohorts (Table 3) which have shown no prognostic value of the CRM status in univariate analysis (213, 214, 262, 264, 265). Since the initial publication of this work a further study using a homogenous cohort of OeC patients who had undergone neoadjuvant chemoradiotherapy (n=104) has been published (261). This showed that a positive CRM did have prognostic value through univariate analysis, however was not an independent prognostic marker (p=0.124).

The reported median frequency of CRM positivity in oesophageal cancer is 42% ranging from 9% to 71% (201, 204, 206-216, 260-264, 266-268). This wide range could be related to the use of different OeC treatment regimens. Thus, 8 studies investigated surgery alone patient cohorts, 1 study investigated neoadjuvant chemotherapy only patients, 3 studies investigated neoadjuvant chemoradiotherapy cohorts only and 9 studies involved cohorts with mixed pre-operative treatment regimens (Table 3). There were also differences between the studies in terms of the different tumour histological subtypes; the 71% CRM positive study involved squamous cell carcinomas (260) while the study reporting a 21% CRM positivity rate investigated mostly adenocarcinomas (84%) (261). However the variation in CRM positivity rates may also be related to the fact that different definitions (RCPath versus CAP) have been used to identify a positive CRM (208, 211-213, 215, 216, 260, 263, 266).

Table 3: Summary of the literature investigating the prognostic relevance of the circumferential resection margin status in patients with oesophageal carcinoma

Author	Years involved	Type	N	Chemo (yes/no)	CRM %	Univariate significance	multivariate significance	RcPath or CAP
Sagar et al (207)	1984-1989	Retro	50	No	50	Yes p<0.05	Not done	RCPath
Dexter et al (206)	1990-1997	Pros	135	No	47	Yes (p<0.015)	Yes (p<0.013)	RCPath
Zafirellis et al (204)	1990-2000	Pros	156	No	42	Yes (p<0.0001)	Not done	RCPath
Khan et al (264)	1982-1996	Retro	329	No	20	No (p=0.57)	N/A	RCPath
Griffiths et al (212)	1994-2003	Retro	249	Yes (13%)	32	Yes (p=0.0001)	Yes (p=0.007)	RCPath
Sujendran et al (211)	1997-2004	Pros	242	Yes (59%)	22	Yes (p=0.032)	Yes (p=0.006)	RCPath
Chao et al (208)	1997-2005	Retro	151	Yes* (100%)	51	Yes (p<0.05)	Not done	RCPath
Scheepers et al (210)	2000-2005	Retro	110	No	38	Yes (p= 0.004)	Yes (p=0.006)	RCPath
Deeter et al (216)	1991-2006	Pros	135	Yes (44%)	61	No (p=0.14)	N/A	Both
Pultrum et al (201)	1997-2006	Pros	98	No	48	Yes (p=0.002)	Not done	Both
Saha et al (209)	2000-2006	Pros	105	Yes (100%)	36	Yes (p<0.001)	Yes (p=0.002)	RCPath
Rao et al (215)	2000-2006	Retro	157	Yes (24%)	50	No (p=0.056)	N/A	RCPath
Sillah et al (266)	1994-2007	Retro	320	Yes (38%)	28	Yes (p=0.015)	Not done	RCPath
Thompson et al (265)	1997-2007	Retro	240	Yes (52%)	35	Yes (p=0.001)	No	RCPath
Mirnezami et al (262)	2000-2007	Pros	314	No	46	Yes (p=0.011)	No	RCPath
Verhage et al (268)	1988-2008	Pros	260	No	67	No (p=0.15)	N/A	Both
Harvin et al (214)	1997-2008	Retro	160	Yes* (100%)	26	No (p=0.84)	n/a	RCPath
Okada et al (260)	1997-2011	Pros	160	Yes (42%)	71	Yes (p=0.014)	No (p=0.570)	Both
O'Farrell et al (213)	2003-2011	Retro	157	Yes (52%)	60	No (p=0.168)	No (p=0.137)	RCPath
Reid et al (263)	1998-2012	Pros	269	Yes (46%)	38	Yes (p<0.001)	Yes (p=0.004)	RCPath
Hulshoff et al (261)	2005-2013	Retro	104	Yes* (100%)	21	Yes (p<0.001)	No (p=0.124)	Both

CRM = circumferential resection margin, N= number of patients. Pros= prospective and Retro= retrospective study. *= studies investigating patients treated with pre-operative chemoradiotherapy.

Studies which examined the prognostic value of the two definitions of a positive CRM have contrasting results (201, 213, 216, 260, 268). The studies by Pultrum et al (201) and O'Farnell et al (213) supported the clinical relevance of the RCPath definition, while Verhage et al (268), Deeter et al (216) and Okada et al (260) concluded that the CAP definition of a positive CRM was a better prognosticator. However, two of these studies compared patients treated by surgery alone (201, 268) and three studies investigated mixed cohorts composed of patients who may or may not have had neoadjuvant treatment (213, 216, 260). At the time of this study, there were no data available comparing the prognostic value of tumour cells present at the CRM with that of tumour cells present within 1 mm but not at the CRM, in a cohort of oesophageal cancer patients treated with chemotherapy followed by radical resection.

This retrospective study aimed to establish whether the prognostic value of tumour cells present directly at the resection margin is different to that where tumour cells are seen within 1mm of the resection margin by using a large cohort of neoadjuvant chemotherapy treated oesophageal cancer patients from two large UK centres

It was hypothesised that although the presence of a positive CRM may have prognostic value in neoadjuvant chemotherapy treated oesophageal cancer it is not an independent prognostic marker as shown in surgery alone studies previously.

2.2 Methods

2.2.1 Patients selection

This study was carried out using a cohort created from databases of oesophagectomies carried out within the Leeds teaching hospitals NHS trust (LTHT) and South Manchester NHS trust. A total of 465 patients who had undergone an Ivor-Lewis oesophagectomy with 2-field lymphadenectomy were assessed for eligibility for inclusion. The patient inclusion and exclusion criteria are set out in Figure 7. Patients

with evidence of metastatic disease were excluded from the study. Patients with a complete pathological response were excluded for the final cohort (n=9) as they had no residual tumour cells remain to assess for CRM status. Although they could have been placed in the CRM –ve group these patients are likely to have the best prognosis with longest survival so could have been the reason for previous findings of CRM being an independent prognostic marker (209, 211). Through discussions between the research group it was decided for this study these patients would be excluded from the final cohort.

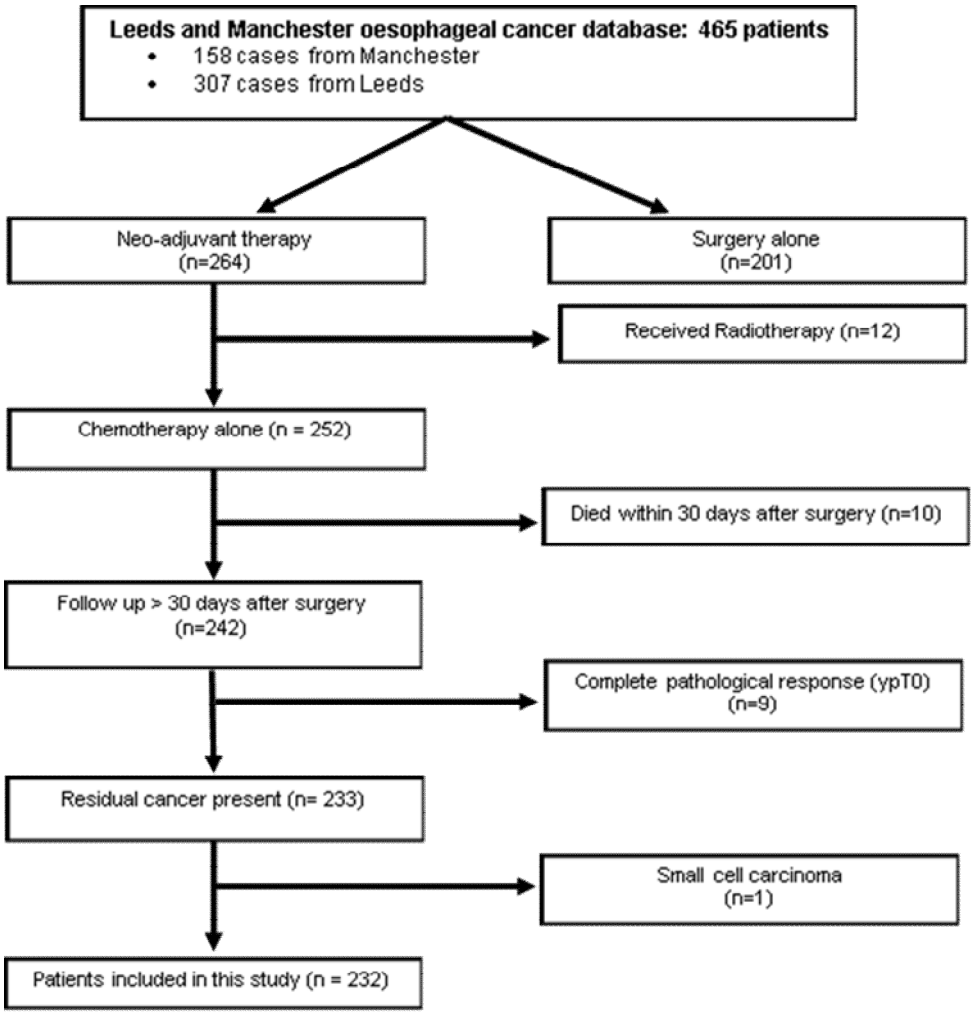


Figure 7: Circumferential resection margin study flow chart.
The flow chart shows how the final cohort was created including exclusion criteria and number of patients excluded at each step.

In total 232 patients who received two cycles of 5-Fluorouracil/ Cisplatin chemotherapy according to OE02 trial regimen (99) followed by surgery, were included in the study. 184 patients were treated in Leeds between 2001 and 2009 and 48 patients were treated in Manchester between 1995 and 2008. The two hospital cohorts were compared to ensure they were comparable in terms of clinicopathological features and survival rates, for details see Table 4.

Table 4: Comparison of the clinicopathological and survival data of the two hospital patient cohorts

	Combined cohort n=232	LTHT cohort n=184	South Man* n=48	
	Number (%)	Number (%)	Number (%)	p value
Gender				
Male	177 (76.3)	139 (75.5)	38 (79.2)	0.600
Female	55 (23.7)	45 (24.5)	10 (20.8)	
Histology				
Adenocarcinoma	183 (78.9)	141 (76.6)	42 (87.5)	
Squamous	45 (19.4)	40 (21.7)	5 (10.4)	0.111
Adenosquamous	4 (1.7)	3 (1.6)	1 (2.1)	
ypT (TNM7)				
T1a	7 (3.0)	4 (2.2)	3 (6.3)	
T1b	15 (6.5)	14 (7.6)	1 (2.1)	
T2	39 (16.8)	26 (14.1)	13 (27.1)	0.073
T3	162 (68.9)	131 (71.2)	31 (64.6)	
T4a/b	9 (3.9)	9 (4.9)	0 (0.0)	
ypN (TNM7)				
N0	70 (30.2)	52 (28.3)	18 (37.5)	
N1	60 (25.9)	50 (27.2)	10 (20.8)	0.473
N2	49 (21.1)	40 (21.7)	9 (18.8)	
N3	53 (22.8)	42 (22.8)	11 (22.9)	
Mortality				
Alive	136 (58.6)	106 (57.6)	30 (62.5)	0.541
Dead	96 (41.4)	78 (42.4)	18 (37.5)	
Cancer specific survival				
Alive or other death	158 (68.1)	126 (68.5)	32 (66.7)	
Cancer death	64 (27.6)	48 (26.1)	16 (33.3)	0.999
Unknown	10 (4.3)	10 (5.4)	0 (0.0)	

*South Man= South Manchester cohort

The majority of cases in the cohort were males (76%) and had ypT3 (70%) tumours. The combined cohort included patients with both adenocarcinomas (n=183) and squamous cell carcinomas (n=45), as well 4 patients who had adenosquamous cancers.

There was no significant difference in age or lymph node status between adenocarcinomas (n=183) and squamous cell carcinoma (n=45) (Table 5).

Table 5: Patient age, total number of lymph nodes and positive lymph nodes by histology

	Total (n=232)	Adeno[1] (n=183)	Squam[2] (n=45)	P value
Age (years)				
Median	**62**	62	61	0.34
Range	**35-78**	35-78	41-75	
Total number of lymph nodes				
Median	**30**	29	34	0.57
Range	**3-82**	3-82	3-70	
Number positive lymph nodes				
Median	**2**	2	1	0.52
Range	**0-63**	0-63	0-15	

[1] adenocarcinoma and [2] squamous cell carcinoma. P values found using Kruskal Wallis testing.

2.2.2 Circumferential resection margin subgroups

Specialist gastrointestinal pathologists in Leeds (Heike Grabsch, HG) and Manchester (Gavin Udall and Susan Pritchard) reviewed all histopathology reports. If the distance of cancer cells from the CRM was not provided in the report, the original Haematoxylin and Eosin (H&E) stained slides were reviewed and the distance of cancer cells from the CRM was measured in mm.

The CRM status was classified into three groups;

- Group A: cancer cells at the CRM (equal to a distance of 0 mm from the margin)
- Group B: cancer cells within 1 mm but not directly at the CRM
- Group C: no cancer cells within 1 mm of the CRM.

2.2.3 Data collection and follow up

The following clinicopathological data were used for analyses: age at diagnosis, gender, ypT and ypN categories according to TNM 7th ed.(139), tumour morphology according to WHO classification (269), total number of lymph nodes examined, total number of positive lymph nodes and nearest distance of viable cancer cells to the circumferential resection margin. Follow-up and mortality data were retrieved from Cancer Registry Information Service database and hospital patient records. Patients were followed up until the end of the study period or death with routine outpatient clinic reviews. LTHT data was collected by Mr Paul Jose (research fellow LTHT) and Manchester data Mr Ahmed Mirza (South Manchester research fellow), before being combined and analysed by the author of this thesis.

2.2.4 Statistical analyses

After the two hospital centres databases were combined statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 17.0 (Chicago, Illinois). The Kruskal-Wallis test was used to establish the relationship between CRM status, tumour morphology, ypT and ypN. The relationship of CRM status and cancer specific survival was determined by the Kaplan-Meier method (270) and differences between groups were tested by the log-rank test. Follow up time was calculated from the day of surgery to patient death or end of study period.

A Cox's proportional hazard model was used for univariate and multivariate analysis. Variables tested in univariate analyses were patient age, gender, histological tumour type, ypT category, ypN category, number of lymph nodes retrieved, number of involved lymph nodes and CRM. Only variables that were significant in univariate survival analysis were included in multivariate analysis. A p value of less than 0.05 was considered to be significant.

2.3 Results

2.3.1 CRM status and clinicopathological features

Thirty-eight (17%) specimens were classified as Group A (cancer cells at CRM), 89 (38%) as Group B (cancer cells within 1 mm but not at CRM) and 105 (45%) as Group C (no cancer cells within 1 mm of CRM). The median follow up of all patients was 1.5 years (range: 0.1 to 9.0 years) and the median survival of patients alive at the end of the study period was 2.5 years (range: 0.2 to 9.0 years). The relationship between clinicopathological features and CRM groups is shown in Table 6.

There was no relationship between any of the investigated clinicopathological features comparing Group A with Group B (RCPath CRM vs CAP CRM). However, a significant difference was found in depth of tumour invasion (ypT) and lymph node status (ypN) when patients in Group A and Group B were combined and compared to those in Group C. Group A and Group B patients had more frequently a higher ypT category and higher ypN category compared to Group C patients *(*p<0.001).

Table 6: Patient characteristics stratified by the circumferential resection margin groupings

	Total (n=232)	**Group A (n=38)**	**Group B (n=89)**		**Group C (n=105)**	
	N (%)	N (%)	N (%)	p value[1]	N (%)	p value[2]
Age (years)						
Median	62	62	62	0.47	61	0.540
Range	35-78	41-76	41-78		35-78	
Number of lymph nodes retrieved						
Median	30	31	30	0.64	31	0.880
Range	3-82	9-64	3-82		3-77	
Number of positive lymph nodes						
Median	2	6	3	0.98	1	***<0.001***
Range	0-63	0-63	0-28		0-16	
Gender						
Male	177 (76)	32 (84)	64 (72)	0.141	81 (77)	0.318
Female	55 (24)	6 (16)	25 (28)		24 (23)	
ypT category						

T1a	7 (3)	0 (0)	0 (0)		7 (7)	
T1b	15 (6)	0 (0)	1 (1)		14 (13)	
T2	39 (17)	1 (3)	5 (6)	0.128	33 (31)	***<0.001***
T3	162 (70)	33 (86)	79 (89)		50 (48)	
T4	9 (4)	4 (11)	4 (4)		1 (1)	
ypN category						
N0	70 (30)	5 (13)	18 (20)		47 (45)	
N1	60 (26)	8 (21)	22 (25)	0.113	30 (29)	***<0.001***
N2	49 (21)	8 (21)	23 (26)		18 (17)	
N3	53 (23)	17 (45)	26 (29)		10 (9)	
Morphology						
Adeno[3]	183(79)	30 (79)	65 (73)		88 (84)	
Squam[4]	45 (19)	8 (21)	22 (25)	0.451	15 (14)	0.196
Adenosq[5]	4 (2)	0 (0)	2 (2)		2 (2)	

Group A: Tumour cells at the circumferential margin; Group B: Tumour cells within 1 mm but not at margin and Group C: No tumour cells within 1 mm of the margin.
[1] Kruskal-Wallis test comparing Group A with Group B,
[2] Kruskal-Wallis test comparing Groups A+B with Group C
[3]Adenocarcinoma, [4]Squamous cell carcinoma, [5] Adenosquamous carcinoma.

2.3.2 CRM status and cancer specific survival

There was no difference in cancer specific survival between Group A (at the margin) and Group B (tumour within 1mm) patients (p= 0.945). However, patients from both groups had significantly poorer survival compared to Group C patients (p= 0.008, Figure 8). Univariate survival showed ypT and ypN were significantly related to patient prognosis and were therefore included in multivariate analysis (Table 7). Multivariate survival analysis showed that CRM status was not an independent prognostic factor (Table 7). Only the lymph node status (ypN category) remained a significant prognostic factor on multivariate analysis (p= 0.003). Although the survival difference between Group B vs Group C was shown to be slightly more significant than between Group A vs Group C, this is likely to be as a result of Group A only having 38 patients compared to Group B (n=89).

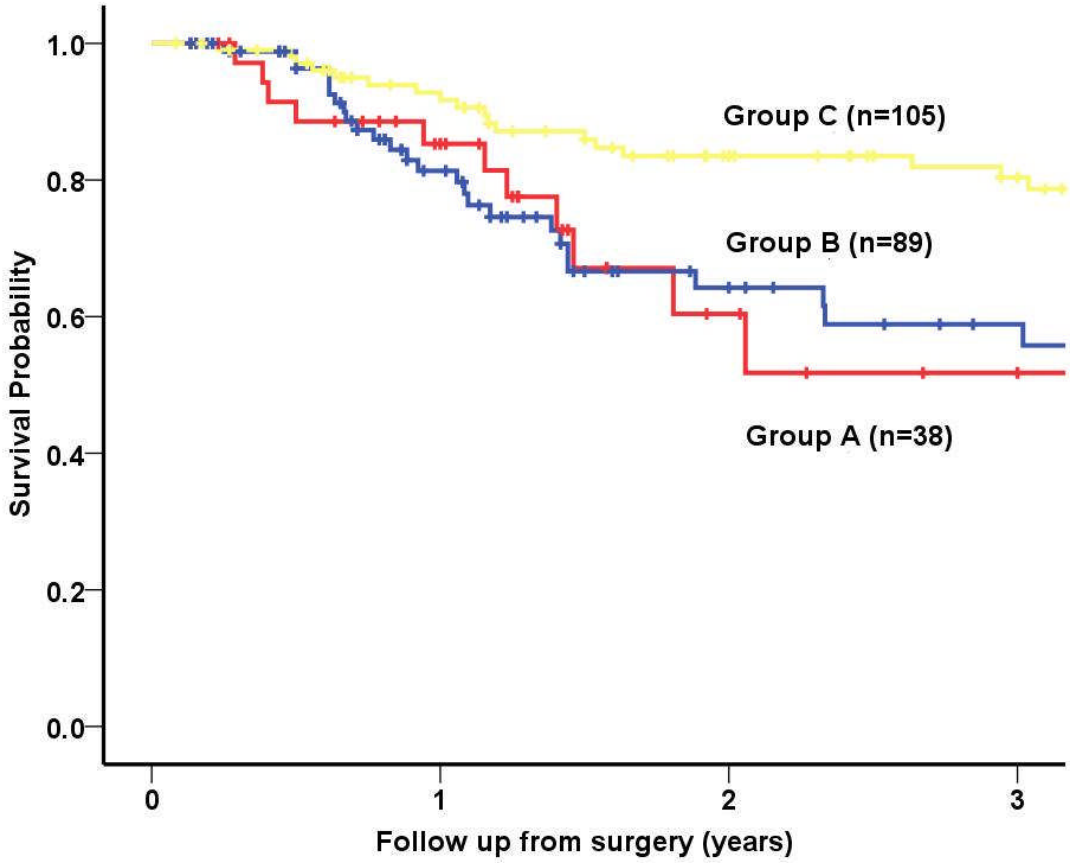


Figure 8: Kaplan Meier cancer specific survival plot stratifying patients by CRM group
Kaplan Meier plot comparing the cancer specific survival probability of Group A (tumour cells at CRM), Group B (tumour cells within 1mm but not at CRM) and Group C (no tumour cells within 1mm). There is a significant difference in survival between patients in Group C compared to both Group A and Group B (Log rank test p= 0.008; Hazard Ratio= 0.45; 95% confidence intervals 0.27 to 0.75).

Table 7: Relationship between clinicopathological variables, CRM status and cancer specific survival

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.01	0.97-1.03	0.744	-	-	-
Gender	1.47	0.78 to 2.75	0.232	-	-	-
Morphology	0.48	0.23 to 1.01	0.053	-	-	-
ypT	1.94	1.27 to 2.98	**0.002**	1.49	0.96 to 2.32	0.078
ypN	1.99	1.57 to 2.54	**<0.001**	1.53	1.45 to 2.05	**0.004**
CRM status						
Group A vs. C	2.20	1.06 to 4.54	**0.034**	0.745	0.33 to 1.70	0.484
Group B vs. C	2.23	1.30 to 3.85	**0.004**	1.30	0.73 to 2.34	0.375
Number of lymph nodes	0.99	0.97 to 1.01	0.205	-	-	-
Number of positive nodes	1.10	1.07 to 1.12	**<0.001**	1.06	1.02 to 1.10	**0.004**

HR= Hazard Ratio, CI= confidence interval

In the subgroup of patients with adenocarcinoma (n=183), Kaplan Meier survival analysis showed a significant difference in survival between patients in CRM groups A+B compared to group C (p=0.01). There was no significant difference in survival between patients in group A and group B (*p*=0.861, Figure 9a).

In the subgroup of patients with squamous cell carcinoma (n=45), Kaplan Meier survival analysis showed no significant difference in survival between patients in CRM groups A+B compared to group C (p= 0.354) and no significant difference in survival between patients in group A and group B (p=0.643, Figure 9b).

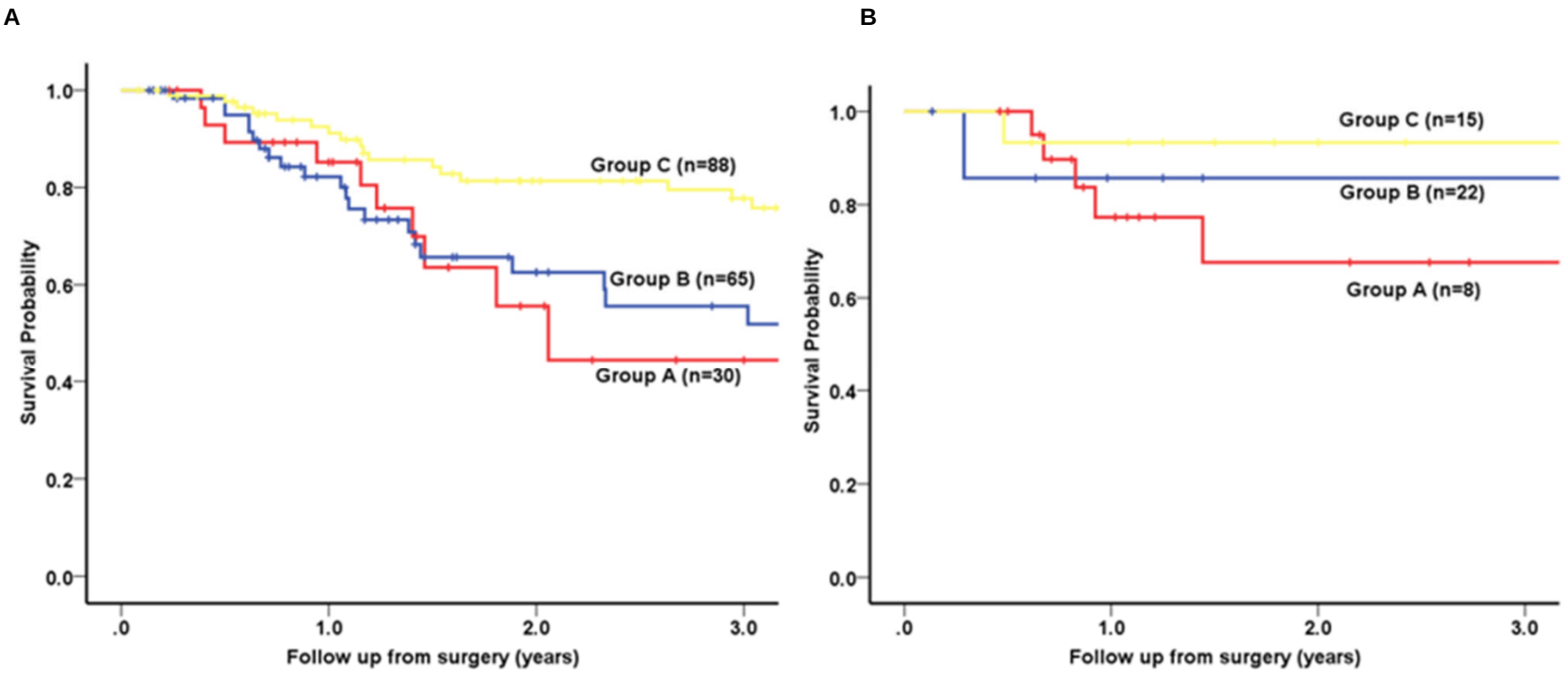


Figure 9: Kaplan Meier survival plots for adenocarcinomas and squamous cell carcinomas stratified by CRM groups.

A. Kaplan Meier plot for patients with adenocarcinoma (n= 183). There is a significant difference in survival between patients in Group C compared to both Group A and Group B (Log rank test p= 0.01; HR 0.61; 95% CI 0.43 to 0.87). There was no significant difference in cancer specific survival between Group A and Group B (p= 0.861).

B. Kaplan Meier plot for patients with squamous cell carcinoma (n=45). There is no significant difference in survival between patients in Group C compared to both Group A and Group B (Log rank test *P*= 0.354; Hazard Ratio= 0.617; 95% confidence intervals 0.22 to 1.74). There was no significant difference in cancer specific survival between Group A and Group B (p=0.643)

Subgroup analysis looking specifically at the patients with ypT3 only disease was done as well as looking just at patients with no evidence of nodal disease (ypN0). Using only ypT3 patients (n=162) showed similar numbers of patients in group A (n=33) and B (n=79). However in this subgroup of patients there were only 50 patients (31%) who were CRM –ve so placed in group C. Although patients in group A and B had worse survival than patients in Group C this was not shown to be statistically significant (p=0.219). There was also no significant difference in survival between patients in groups A and B (p=0.894) (Figure 10).

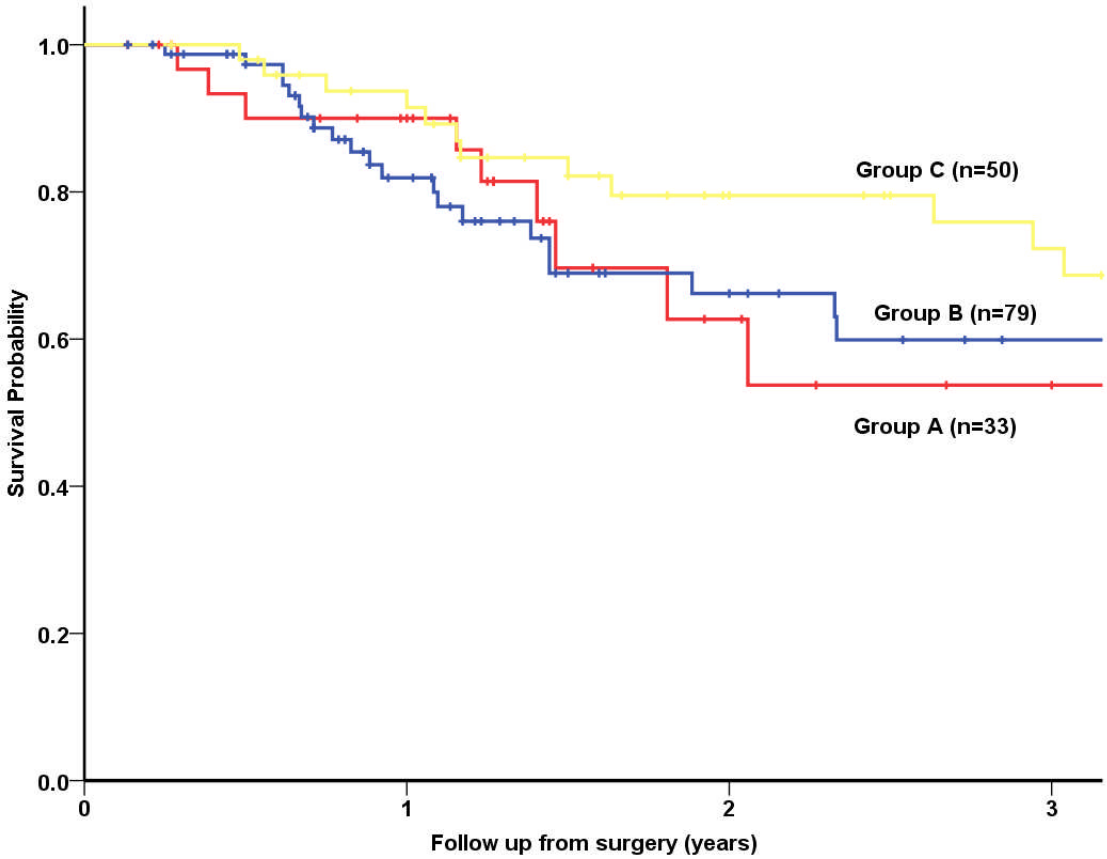


Figure 10: Kaplan Meier cancer specific survival for the ypT3 patients by CRM group
Although patients in group A and B had visible evidence of poorer survival probability this was not shown to be significantly different to the Group C patients when only ypT3 patients were included (p=0.219)

The ypN0 patients were also looked at individually (n=70) there were 5 (7%) patients who had tumour at the CRM margin (Group A) and 18 patients (26%) had tumour withing 1mm but not at the margin (Group B). Log rank testing shows that there is no significant difference

in survival between patients in group A and B than patients in Group C (p=0.701). There was also no significant difference in survival between patients in groups A and B (p=0.593). However in patients with evidence of nodal disease (ypN1-3) there was also no significant difference in survival between patients in groups A and B compared to group C (p=0.049).

2.4 Discussion

Circumferential resection margin (CRM) status has been recognised as an important prognostic factor for patients with rectal cancer (271-279). In rectal cancer, a 1 mm cut-off is currently used to define a positive CRM (271). Initial studies in oesophageal cancer patients treated by surgery alone adopted the 1 mm cut-off and showed that the presence of cancer cells within 1 mm of the CRM was related to patient survival in univariate (207) and multivariate analysis (206). A number of subsequent studies using the within 1mm definition confirmed this finding in univariate analysis (201, 204, 208, 266) and multivariate analysis (209-212). However, other studies were unable to show that CRM status is an independent prognostic factor (213, 262, 265), or failed to identify CRM status as being significantly related to prognosis at all (214, 264).

The ongoing controversy regarding the prognostic value of the CRM in oesophageal cancer patients may be related to the fact that the investigated patient cohorts received different treatment regimens. Eight published studies involved a cohort of surgery alone (201, 204, 206, 207, 210, 262, 264, 268), nine studies involved a mixture of patients treated with neoadjuvant chemotherapy or surgery alone (211-213, 215, 216, 263, 265, 266), three studies included patients treated with pre-operative chemoradiation (208, 214, 260). Prior to our own study, one previous study had investigated a cohort of neoadjuvant chemotherapy patients (209). Evidence shows that patients who have undergone neoadjuvant chemotherapy have lower rates of R1 resections (84, 280). Furthermore, while most authors have used the presence of cancer cells within 1 mm as the primary definition of a positive

CRM (204, 206, 207, 212, 264), some have used the presence of cancer cells directly at the margin to define a positive CRM (216, 268) which along with heterogeneous patient treatment groups may explain the wide range of reported CRM positivity rates: median 42%, range 9% to 71% (201, 204, 206-216, 260-264, 266-268).

The current study was the first to compare the prognostic value of the RCPath definition (within 1mm) and the CAP definition (at the margin) of a positive CRM in a cohort of oesophageal cancer patients treated with neoadjuvant chemotherapy followed by surgery. In the current study, 54% of specimens were classified as CRM positive using the RCPath definition of tumour cells within 1 mm of the CRM and 17% had a positive CRM using the CAP definition.

In the current study, patients treated with neoadjuvant chemotherapy and no viable cancer cells within 1 mm of the CRM had a significantly better survival than those where cancer cells were present within 1 mm of the CRM. There was no difference in the survival probability of patients with cancer cells at the margin compared to those with cancer within 1 mm of the CRM. Thus, the results do not support the recently advocated view that the CAP CRM definition (i.e. cancer cells at the margin) is more accurate for prognostic stratification of patients (216, 260, 268). The same results were obtained when the analyses were restricted to patients with adenocarcinomas. Grouping patients by CRM status had no significant relationship to survival in patients with squamous cell carcinomas which is most likely to be due to the small overall number of patients in this subgroup (n=45). This part of the study is in contrast to previous studies examining squamous cell carcinoma of the oesophagus (208, 260). Studies including only patients with oesophageal adenocarcinoma have contrasting results regarding the prognostic value of CRM, with some showing it is a prognostic marker (209, 210) and other saying it does not have a prognostic value (214, 268). However, it is difficult to compare studies given the heterogeneity of the treatments patients received and the different definitions of a CRM margin used.

In the current patient cohort, the prognostic value of the CRM status was no longer apparent in multivariate analysis where only lymph node status (ypN) proved to be an independent prognostic factor. This finding is in contrast to a number of CRM studies which have shown CRM to be an independent prognostic marker (206, 210-212, 263), including the only other study in patients treated with pre-operative chemotherapy (209). However, the current study cohort included more than double the number of patients and included squamous cell carcinomas as well as adenocarcinomas, giving a very different cohort group than that used in the previous neoadjuvant chemotherapy study (209). There were also differences in variables included in the multivariate analysis as here only variables significant on univariate analysis were included in the multivariate study. It's difficult to compare this current study to others which included a mixed patient treatments in the study cohort (212, 263) or the studies which involved patients who had only undergone surgery alone (206, 210). Although there is no widely accepted evidence to confirm neoadjuvant therapy reduces CRM rates, it has been shown to reduce the number of overall R1 resections in OeC through downstaging of the tumour itself (94, 281).

This work has some limitations. This was a retrospective analysis using data from two different centres. However as the ypT category, ypN category, cancer mortality and survival did not differ between the two centres, it was felt that the datasets from the two hospitals could be combined (Table 4). The study had a relatively short follow-up period, and thus analyses with longer follow up might show different results. Due to the retrospective nature of the current study, neither time to recurrence or pattern of tumour recurrence or primary tumour regression data were available, so we were unable to investigate the relationship between CRM status and these variables.

In conclusion, this work demonstrated that oesophageal cancer patients treated by pre-operative chemotherapy with cancer cells at the CRM or within 1 mm of the CRM of the resected specimen have a significantly worse survival than patients with no cancer cells

within 1 mm of the margin. This work therefore supports the use of the ‘cancer within 1 mm of the CRM’ rule e.g. the RCPath definition (200), to define a positive CRM.

Furthermore, our results suggest that post-operative lymph node status is the most important independent factor in determining prognosis in oesophageal cancer patients treated with neoadjuvant chemotherapy, more important than the circumferential resection margin status. However, there is currently still uncertainty whether a better outcome can be achieved with a three field or a two field lymphadenectomy(282). It is possible that also the new TNM7 classification including more ypN categories (2 previously in TNM6 now 4 in TNM7) has had an influence on better stratification of patient prognosis (147, 283).

Since the original publication of this work, a meta-analysis of all data published on CRM status in OeC has been carried out. This analysis pooled 14 studies (including the work from this study) and included 2433 patients who had undergone a potential curative oesophagectomy for oesophageal cancer (203). It showed that CRM involvement was less frequent in patients from neoadjuvant chemotherapy studies compared to surgery alone studies using either the RCPath definition (40.1% vs. 34.3) or the CAP CRM definition (22.2% vs. 11.2%). A significantly poorer survival was found in patients who had tumour within 1mm compared to those with no microscopic evidence of tumour within 1mm ($p<0.001$), in keeping with the findings in this study. Patients with tumour involvement at the CRM (CAP definition) were shown to be a higher risk group with larger OR values in both 3 and 5 year survival. However as patients with tumour within 1mm but not at the margin (Group B in this study) also had a significantly worse survival compared to patient with no tumour involvement within 1mm, the meta-analysis in agreement with this study concluded the RCPath definition for CRM involvement was more comprehensive (203).

3. THE OE02 THESIS COHORT'S CLINICOPATHOLOGICAL CHARACTERISTICS AND SURVIVAL

3.1 Background- OE02 MRC trial

The MRC OE02 trial investigated the potential benefit of neoadjuvant 2 drug chemotherapy in the management of OeC patients with resectable disease and it's results changed practice in the UK (99). Prior to the OE02 trial, the majority of UK patients with resectable disease had a surgical resection and 3 year overall survival between 20-30% (284-286) and 5 year survival being reported as ranging between 0.5-7% (287, 288).

The OE02 trial was an intention-to-treat trial which recruited 802 patients in the UK and the Netherlands during 1992 to 1998. Patients were randomised to either the surgery alone or neoadjuvant chemotherapy (two cycles of Cisplatin and 5-Fluorouracil) followed by surgery treatment groups. Included patients had squamous cell carcinoma, adenocarcinoma and undifferentiated tumours found anywhere along the oesophagus but below the cricoid. Patients with distant metastases (including cervical lymph node involvement) were excluded. 402 patients were randomised to the surgery alone group (S), of which 386 went on to have surgery, and 400 patients were allocated to the pre-operative chemotherapy group (CS) of which 344 actually completed at least 1 or 2 cycles and subsequently underwent surgery (99). The primary outcome measure used was patient survival from time of randomisation. This was the largest ever trial carried out to compare these two treatment groups and 93% of patients were followed for five years, with 83% of patients followed up for at least 10 years. The chemotherapy cycles and consequent toxic side effects may have contributed to the higher rate of deaths before surgery in the CS group compared to the S group (3% vs. 1%).

Tumours resected in the CS group were significantly smaller (p=0.001) and with less lymph node involvement than tumours from the S group.

The initial survival data published in 2002 showed a difference in the two groups median overall survival rates with patients in the CS group surviving a median of 16.8 months compared to patients in the S group whose median survival was 13.3 months. On statistical analysis it was demonstrated that CS group had a significantly better overall survival (p=0.04) and disease free survival (p=0.0014) than those who had underwent surgery alone (99). Longer term survival figures published subsequently in 2009 confirmed the survival benefit of neoadjuvant chemotherapy (94).

Although the primary outcome analysis from OE02 has been published, no data has been published looking at possible prognostic markers for patients in both treatment arms. Using material from resection specimens from this trial gives an opportunity to analyse potential new prognostic markers from a unique trial that changed practice and thus will most likely be the last trial where patients with oesophageal cancer are randomised to surgery alone. Although the trial was obviously powered to show a clinical difference and thus may not have enough power to demonstrate a biomarker difference, using material from patients from a randomised control trial is clearly preferable to the use of local hospital material due to inherent bias.

3.2 Methods

3.2.1 OE02 material local collection

OE02 trial material and pathology data were requested by Dr H Grabsch affiliated with Leeds Institute of Cancer and Pathology (LICAP), University of Leeds. The material for translational research was funded by a project grant from Cancer Research UK. H&E stained slides were reviewed to confirm the histopathological data, fill in missing data and establish the tumour regression grade by histopathologist Dr N West (NW) and Dr H.I Grabsch (HG). Tumour regression grading was not recorded at the time of the initial OE02 trial. All tissue slides

were scanned and stored on a networked server for viewing as well as analysis. Tissue blocks where used to create tissue microarray slides for immunohistochemistry investigations discussed later on.

3.2.2 Patients

Although 802 cases were included in the original OE02 trial, material from 508 resection cases was retrieved by the LICAP.

3.2.3 Statistical analysis

Statistical analysis was performed using the computer program Statistical Package for the Social Sciences (SPSS) 21.0 (Chicago, Illinois). Clinicopathological features between the 508 OE02 trial cases from which material was received and the cases not received from the OE02 trial were compared. Follow up from randomisation to patient death or end of study period was used for survival analysis and survival plots were constructed using the Kaplan-Meier method (270) testing differences between groups by the log-rank test.
Mann-Whitney non-parametric testing was used to compare age, gender, histological subtype, grade of differentiation, T category, N category, blood vessel invasion, lymphatic vessel invasion and Mandard tumour regression grading. A Cox's proportional hazard model was used for univariate and multivariate analysis. Variables tested in univariate testing were patient age, gender, histological subtype, grade of differentiation, T category, N category, blood vessel invasion, lymphatic vessel invasion and Mandard tumour regression grading. Only variables that were significant in univariate survival analysis were included in multivariate analysis. A p value of less than 0.05 was considered to be significant.

3.3 Results

3.3.1 Cohort comparison

Table 8 shows a comparison of the 508 case cohort for which material was received by LICAP and the not received OE02 cases (n=294). Patients treatment arm is determined by

their actual treatment not by the treatment arm the patient was originally assigned to on randomisation (therefore eight cases originally assigned to chemotherapy followed by surgery group that actually only underwent surgery alone were reallocated). Original histology was taken from initial biopsy results. T and N categories were taken from original pathology reports if available. The statistical analyses showed that the clinicopathological features of the received OE02 cases are similar to the cases not received (Table 8).

Table 8: Clinicopathological data for OE02 trial cases for which material was received and those for which no material was received

	Overall OE02 cohort (%) n=802		LICAP received cohort (%) n=508		Cases not received n=294		
	Number	%	Number	%	Number	%	p value
Age							
Median	62.8		62.7		62.9		0.986
Range	30.0-84.1		30.0-83.1		35.5-84.1		
Gender							
Male	603	75.2	382	75.2	221	75.2	0.993
Female	199	24.8	126	24.8	73	24.8	
Treatment arm							
Chemo+ surgery	391	48.8	257	50.6	134	45.6	0.064
Surgery alone	411	51.2	251	49.4	160	54.4	
Original Histology							
Adenocarcinoma	533	66.5	348	68.5	185	62.9	0.823
Squamous	247	30.8	153	30.1	94	32.0	
Other	21	2.6	7	1.4	14	4.8	
Unknown	1	0.1	0	0.0	1	0.3	
T category (TNM6)							
T0	27	3.4	20	3.9	7	2.4	0.638*
T1	53	6.6	40	7.9	13	4.4	
T2	242	30.2	179	35.2	63	21.4	
T3	334	41.6	254	50.0	80	27.2	
T4	0	0.0	0	0.0	0	0.0	
No resection	120	15.0	0	0.0	120	40.8	
Unknown	26	3.2	15	2.4	11	3.7	
N category (TNM6)							
N0	261	32.5	205	40.4	56	19.0	0.194*
N1	389	48.5	287	56.5	102	34.7	
No resection	120	15.0	0	0.0	120	40.8	
Unknown	32	4.0	16	3.1	16	5.4	
R1 Resection							
Yes	197	24.6	143	28.1	54	18.4	0.293
No	423	52.7	322	63.4	101	34.3	
No resection	120	15.0	0	0.0	120	40.8	
Unknown	62	7.7	43	8.5	19	6.5	

A p value between the variables is calculated using Mann-Whitney test. * These p values were calculated with the unknown cases excluded as they led to inaccurate representation of the distributions of N and T category.

3.3.2 Survival comparison between the cohorts

There was a longer median survival in the thesis OE02 trial cohort compared to the rest of the OE02 trial cases not received by LICAP (1.6 years vs. 0.8 years median length to last follow up or death from randomisation). This is to be expected as all patients whose material was received by Leeds must by definition have undergone surgery so no cases where the patient did not have surgery or did not survive long enough for surgery were excluded from my cohort (40.8% of cases not received did not have an attempted curative resection). This also would have caused the greater proportion of overall deaths in the cases not received (86.4%) compared to the thesis cohort (78.9%).

Kaplan Meier survival analysis shows similar distribution between the two treatment arms, in both cancer specific and overall survival using the time from randomisation (Figure 11 and Figure 12). On log rank (mantel-cox) assessment there is no significant difference in survival between the two treatment groups of patients in regards both overall and cancer specific survival in the not received OE02 cohort (n=294 p=0.626 and p=0.510 respectively). In the same test in the thesis cohort of the 508 cases received there was also no statistically significant difference between the two treatment arms (overall survival p=0.084 and cancer specific p=0.149).

The two treatment group's overall and cancer specific survival was also stratified according to if they were received or not received by LICAP (Figure 13 and Figure 14). The Kaplan Meier plots show patients whose trial material was received and hence included in this thesis had a significantly better survival than those patients who material was not received or did not undergo surgery for any resection material to be available

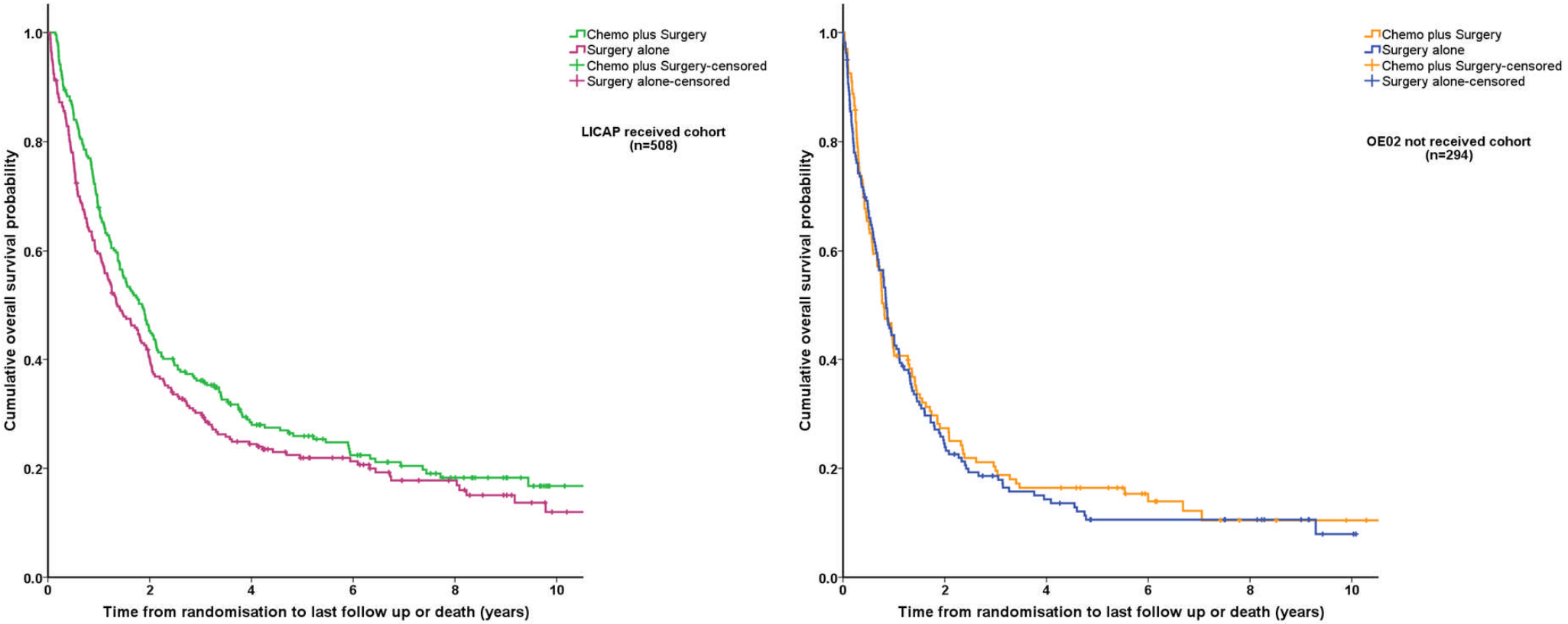


Figure 11: Kaplan-Meier overall survival plots of the OE02 received and not received cohort of patients.
Kaplan Meier survival plots showing the overall survival in both cohorts, with the received LICAP thesis cohort (n=508) to the left (green/pink plots) and the non-received case cohort (n=294) to the right (orange/blue plots). The log rank comparisons show no significant difference in overall survival between both treatment groups in either cohort (received cohort p=0.084 and non-received p=0.626)

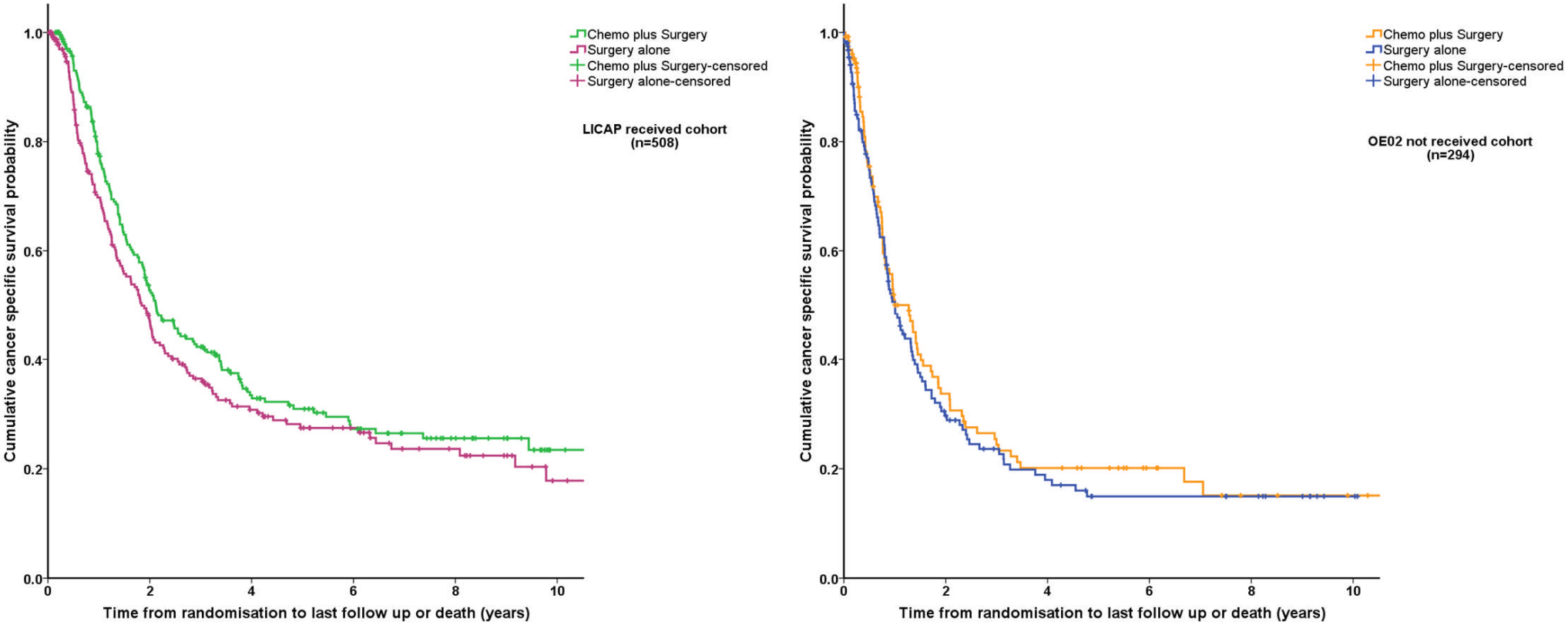


Figure 12: Kaplan-Meier cancer specific survival plots of the OE02 received and not received cohort of patients

Kaplan Meier survival plots showing the cancer specific survival in both cohorts, with the received LICAP thesis cohort (n=508) to the left (green/pink plots) and the non-received case cohort (n=294) to the right (orange/blue plots). The log rank comparisons show no significant difference in cancer specific survival between both treatment groups in either cohort (received cohort p=0.149 and non-received p=0.510).

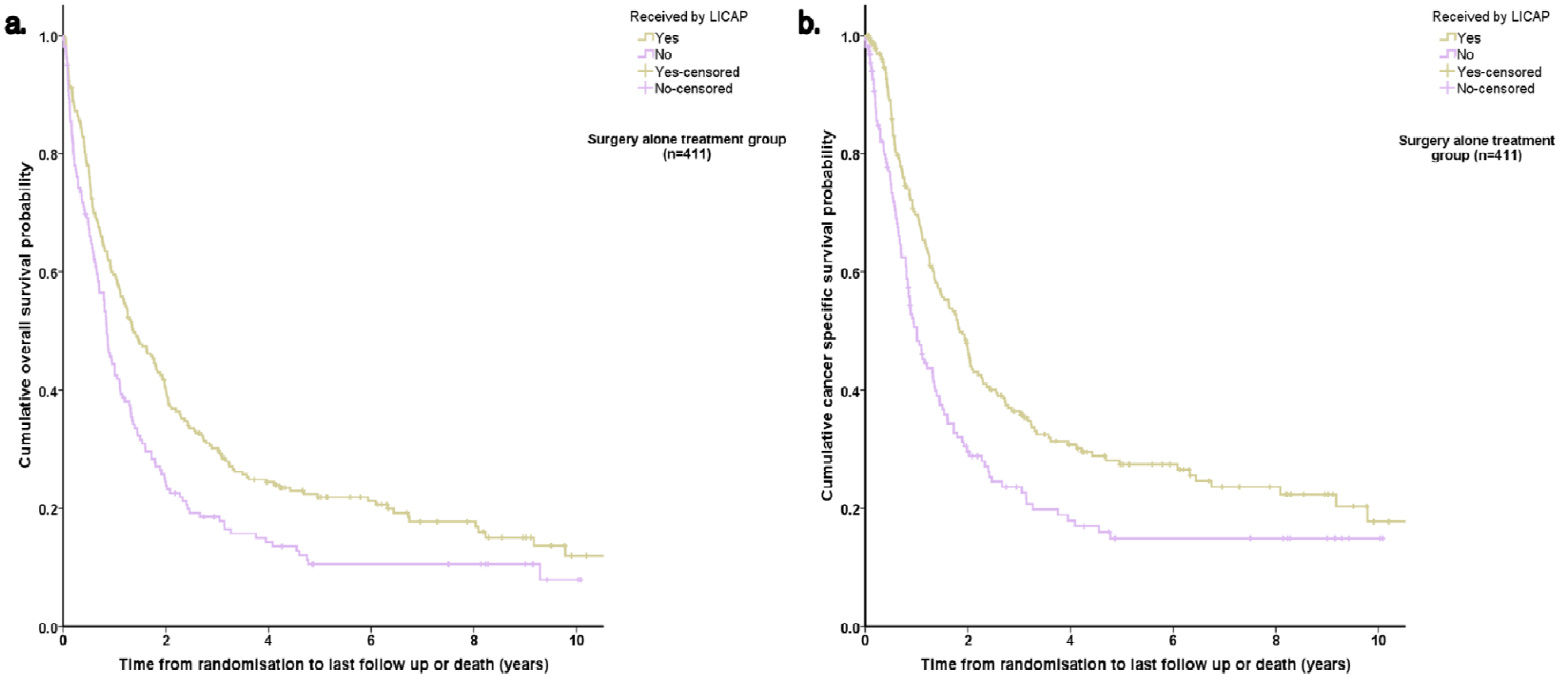


Figure 13: Kaplan Meier overall and cancer specific survival plots for the surgery alone treatment group stratified by whether cases were received or not received for inclusion in thesis

The two plots show the difference in a.) Overall survival; b.) Cancer specific survival for patients from the surgery alone treatment group divided by whether they were received by LICAP (n=251) so included in the thesis and those cases not received (n=160). For both overall and cancer specific survival there is a significantly better survival shown in cases from the received cohort ($p<0.001$).

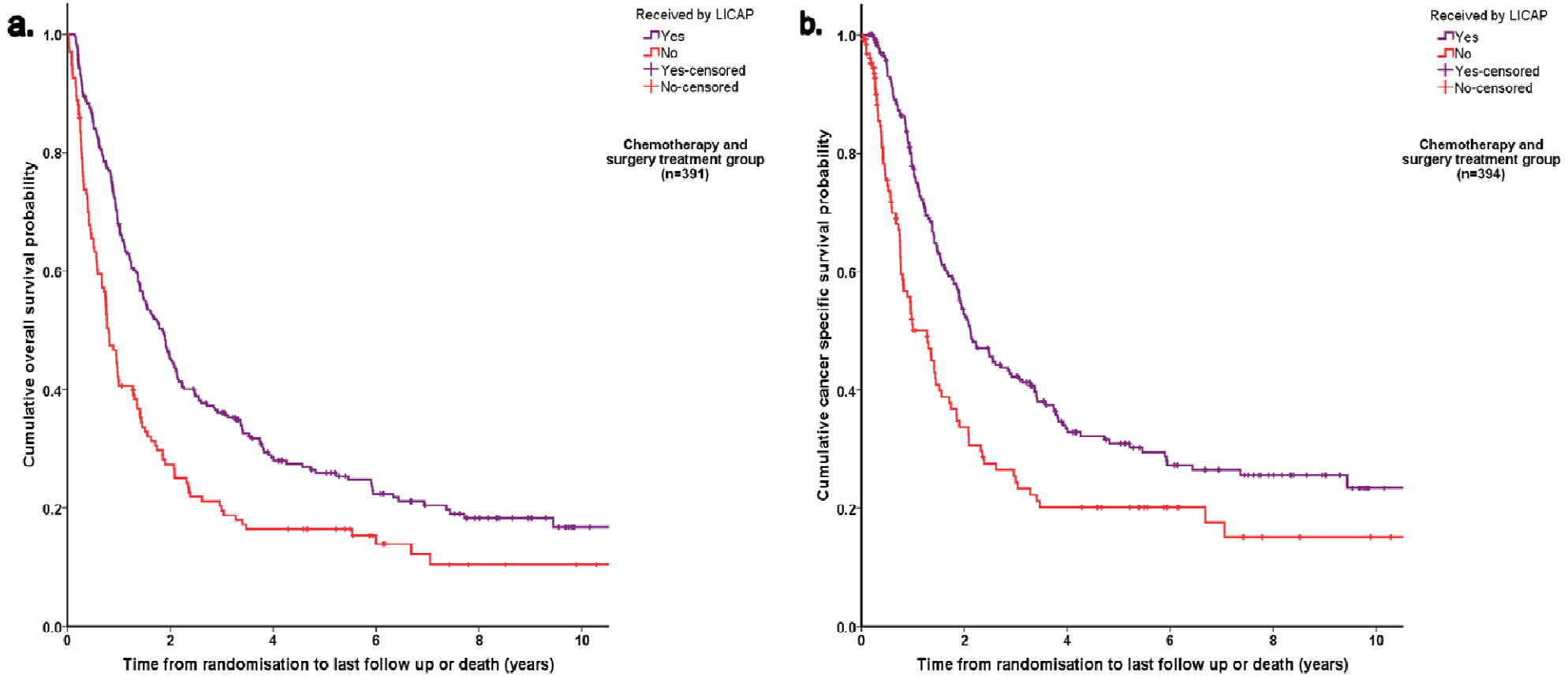


Figure 14: Kaplan Meier overall and cancer specific survival plots for the chemotherapy + surgery treatment group stratified by whether cases were received or not received for inclusion in thesis

The two plots show the difference in a.) Overall survival; b.) Cancer specific survival for patients from the chemotherapy + surgery treatment group divided by whether they were received by LICAP (n=257) so included in the thesis and those cases not received (n=134). For both overall and cancer specific survival there is a significantly better survival shown in cases from the received cohort ($p<0.001$).

3.3.3 Received cohort added pathological features

Once the case cohort was shown to be representative the material received was reviewed along with the original pathology reports to firstly check for concordance with the pathological data but also to establish new pathological data (work done by NW). The full clinicopathological demographics of the thesis cohort after these reviews is summarised in Table 9 and also sub-divided by treatment arm. Mann Whitney U test analysis demonstrates a significant relationship between treatment group and pT category, pN category, presence of lymphatic or blood vessel involvement and Mandard grading (Table 9). There was no statistically difference in age, gender, tumour differentiation or tumour histology between the two treatment arms.

Table 9: Received cohort clinicopathological data by treatment arm.

	Received cohort n=508	*Chemo+ Surg n=257	Surgery only n=251	
	Number (%)	Number (%)	Number (%)	p value
Age				
Median	62.71	62.4	63.6	0.242
Range	30.0-83.2	36.4-83.1	30.0-80.7	
Gender				
Male	382 (75.2)	200 (77.8)	182 (72.5)	0.166
Female	126 (24.8)	57 (22.2)	69 (27.5)	
Histology				
Adenocarcinoma	345 (67.3)	173 (67.3)	172 (68.5)	0.583
Squamous	133 (24.8)	68 (26.5)	65 (25.9)	
Other	18 (3.6)	8 (3.1)	10 (4.0)	
No residual tumour	11 (4.3)	8 (3.1)	3 (1.2)	
Unknown	1 (0.2)	0 (0.0)	1 (0.4)	
Differentiation (Predominant)				
Poor	215 (42.3)	101 (39.3)	114 (45.4)	0.906
Moderate	222 (43.7)	117 (45.5)	105 (41.8)	
Well	44 (8.7)	21 (8.2)	23 (9.2)	
Unknown	16 (3.2)	10 (3.9)	6 (2.4)	
No residual	11 (2.2)	8 (3.1)	3 (1.2)	
T category (TNM6)				
T0 (No residual)	11 (2.2)	8 (3.1)	3 (1.2)	***0.031***
T1	50 (9.8)	27 (10.5)	23 (9.2)	
T2	57 (11.2)	33 (12.8)	24 (9.6)	
T3	376 (74.0)	184 (71.6)	192 (76.5)	
T4	12 (2.4)	4 (1.6)	8 (3.2)	
Unknown	2 (0.4)	1 (0.4)	1 (0.4)	
N category (TNM6)				
N0	209 (41.1)	121 (47.1)	88 (35.1)	***0.006***
N1	299 (58.9)	136 (52.9)	163 (64.9)	
R1 resection status				
Yes	140 (27.6)	70 (27.2)	70 (27.9)	0.810
No	322 (63.4)	165 (64.2)	157 (62.5)	
Unknown	46 (9.1)	22 (8.6)	24 (9.6)	
Lymphatic invasion				
Yes	161 (31.7)	61 (23.7)	100 (39.3)	***0.001***
No	307 (60.4)	177 (68.9)	130 (52.5)	
Suspicious	37 (7.3)	19 (7.4)	18 (7.2)	
Unknown	3 (0.6)	0 (0.0)	3 (1.2)	
Blood vessel invasion				
Yes	59 (11.6)	16 (6.3)	43 (17.1)	***<0.001***
No	412 (81.1)	227 (88.3)	185 (73.7)	
Suspicious	35 (6.9)	14 (5.4)	21 (8.4)	
Unknown	2 (0.4)	0 (0.0)	2 (0.8)	
Mandard tumour regression grade**				
TRG1	22 (4.3)	15 (5.8)	7 (2.8)	***<0.001***
TRG2	12 (2.4)	10 (3.9)	2 (0.8)	
TRG 3	51 (10)	36 (14.0)	15 (6.0)	
TRG 4	170 (33.5)	96 (37.4)	74 (29.5)	
TRG 5	251 (49.4)	99 (38.5)	152 (60.6)	
Unknown	2 (0.4)	1 (0.4)	1 (0.4)	

*chemo+surg= chemotherapy followed by surgery treatment arm patients. **Mandard grading; TRG 1= complete regression, TRG2= few residual tumour cells, TRG3= predominantly fibrosis, TRG4= predominantly tumour cells and TRG5= no evidence regression.

3.3.4 Survival analysis

Cox regression analysis was done to assess whether patient age, gender, histological subtype, grade of tumour differentiation, T category, N category, lymphatic invasion or blood vessel involvement were related to patient survival in the OE02 received cohort. Time from randomisation to last follow up or death was used for survival analysis.

In the surgery alone patient group, age, R1 status, T and N category as well as lymphatic vessel involvement were related to overall survival. There was no relationship with prognosis when patients were stratified for gender, histological subtype or blood vessel involvement (Table 10). Multivariate analysis showed that only patient age, N category, R1 status and the presence of lymphatic involvement were independent prognostic markers for overall survival.

Table 10: Cox regression overall survival analysis in the surgery alone group (n=251).

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.02	1.01 to 1.04	0.006	1.02	1.01 to 1.04	***0.019***
Gender	0.82	0.60 to 1.11	0.199	-	-	-
Histology	1.03	0.85 to 1.25	0.767	-	-	-
Differentiation	1.00	1.00 to 1.00	0.573	-	-	-
pT category	1.47	1.20 to 1.82	<0.001	1.24	0.97 to 1.58	0.070
pN category	2.20	1.62 to 3.00	<0.001	1.85	1.33 to 2.57	**0.007**
R1 status	0.46	0.34 to 0.63	<0.001	0,58	0.42 to 0.80	***0.001***
Lymphatic involvement	1.41	1.15 to 1.72	0.001	1.32	1.06 to 1.64	***0.013***
Blood vessel involvement	1.20	0.98 to 1.47	0.074	-	-	-

In the chemotherapy followed by surgery treatment group, cox regression analysis for overall survival showed prognostic value for patient age, ypT category, ypN category, R1 status lymphatic involvement, blood vessel involvement and Mandard tumour regression grading. However, on multivariate analysis only age, ypT category, ypN category, R1 status and blood vessel involvement were identified as independent prognostic markers (Table 11).

Table 11: Cox regression overall survival analysis in the chemotherapy and surgery alone group.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.02	1.00 to 1.03	0.013	1.02	1.00 to 1.04	***0.0012***
Gender	0.85	0.60 to 1.20	0.346	-	-	-
Histology	0.92	0.77 to 1.09	0.315	-	-	-
Differentiation	1.00	1.00 to 1.00	0.663	-	-	-
ypT category	1.64	1.45 to 1.98	<0.001	1.43	1.16 to 1.77	***0.001***
ypN category	2.01	2.50 to 2.68	<0.001	1.63	1.20 to 2.22	**0.002**
R1 status	0.45	0.33 to 0.61	<0.001	0.62	0.44 to 0.86	**0.004**
Lymphatic involvement	1.34	1.09 to 1.65	0.005	1.16	0.92 to 1.45	0.208
Blood vessel involvement	1.59	1.24 to 2.03	<0.001	1.40	1.08 to 1.81	***0.010***
Mandard Grade	1.24	1.08 to 1.42	0.002	1.02	0.87 to 1.20	0.818

Cox regression analysis performed using cancer specific survival as the outcome measure by treatment arm showed no relationship with age, gender, histological subtype or grade of tumour differentiation in either treatment group. The ypT category,

ypN category, R1 status, lymphatic invasion and blood vessel invasion showed prognostic value in either treatment arm (Table 12 and Table 13). Mandard tumour regression grade was only assessed in the chemotherapy followed by surgery group and was found to be related to cancer specific survival. In patients from the surgery alone group T category, N category, R1 status and lymphatic involvement were identified as independent prognostic markers to cancer specific survival (Table 12).

Table 12: Cox regression analysis for cancer specific survival in the surgery alone group.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.01	1.00 to 1.03	0.125	-	-	-
Gender	0.73	0.51 to 1.04	0.084	-	-	-
Histology	1.02	0.81 to 1.28	0.869	-	-	-
Differentiation	1.00	1.00 to 1.00	0.702	-	-	-
pT category	1.85	1.40 to 2.43	<0.001	1.43	1.04 to 1.97	***0.027***
pN category	2.92	2.01 to 4.23	<0.001	2.15	1.44 to 3.22	***<0.001***
R1 status	0.40	0.29 to 0.57	<0.001	0.51	0.56 to 0.73	***<0.001***
Lymphatic involvement	1.60	1.28 to 2.01	<0.001	1.47	1.14 to 1.88	***0.003***
Blood vessel involvement	1.31	1.05 to 1.63	0.017	1.10	0.87 to 1.38	0.441

Table 13 shows Cox univariate and multivariate analysis for cancer specific survival in the chemotherapy followed by surgery group. Multivariate analysis identified ypT category, ypN category, R1 status and blood vessel involvement as independent prognostic marker. Mandard tumour regression grading was not an independent prognostic marker on multivariate analysis

Table 13: Cox regression analysis for cancer specific survival in the chemotherapy and surgery group.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.02	1.00 to 1.03	0.059	-	-	-
Gender	0.82	0.56 to 1.21	0.318	-	-	-
Histology	0.83	0.67 to 1.02	0.068	-	-	-
Differentiation	1.04	0.93 to 1.16	0.537	-	-	-
ypT category	2.00	1.56 to 2.57	<0.001	1.67	1.28 to 2.17	***<0.001***
ypN category	2.57	1.83 to 3.60	<0.001	1.92	1.35 to 2.72	***<0.001***
R1 status	0.38	0.30 to 0.54	<0.001	0.59	0.40 to 0.85	**0.005**
Lymphatic involvement	1.37	1.09 to 1.73	0.008	1.18	0.91 to 1.52	0.223
Blood vessel involvement	1.36	1.16 to 2.59	<0.001	1.50	1.14 to 1.98	***0.004***
Mandard Grade	1.33	1.13 to 1.55	<0.001	1.08	0.89 to 1.31	0.530

3.4 Discussion

The OE02 trial when its initial results were published in 2002 (99) and the subsequent follow up results published in 2009 (94) changed practice in the UK to include neoadjuvant chemotherapy in the gold standard treatment for locally advanced OeC. It is unlikely there will ever by another trial which includes randomisation of patients to surgery alone for OeC as the results identified a significant survival benefit in patients who had neoadjuvant chemotherapy compared to those who underwent surgery alone (9% benefit in survival) (99). This chapter has shown that the cohort created from received cases at LICAP from the OE02 trial was comparable to the cohort of patients not received. The two cohorts have as demonstrated in table 6 have an identical

gender split (75.2% male: 24.8% female), similar median ages (62.7 in the received cohort vs 62.9 in the not received cohort) and both cohorts involved predominance of adenocarcinomas (68.5 in received and 62.9 in non-received cases). Non parametric tests show no significant difference in any of the clinic-pathological variables in the cases received and not received from the OE02 cohort. This is an important finding as it suggests that the cohort used for this thesis work can be taken as representative of the OE02 trial.

Although the primary outcome analysis from OE02 has been published this is the first data looking at possible prognostic markers for patients in both treatment arms of this trial. Patient age, gender, tumour histological type and grade of differentiation were not shown to have any prognostic value in cancer specific survival for either treatment group. From the received cases cox regression analysis has shown (y)pT, (y)pN category and R1 resection status as independent prognostic markers in the OE02 cohort for cancer specific survival regardless of the treatment group. This finding is in keeping with other recent studies which have examined for clinicopathological variables associated with poor prognosis in resected OeC with (205, 289-291). An R1 resection indicates microscopic evidence of tumour cells at the resection margin and as well as in this cohort has been shown previously to be an independent prognostic marker to survival (204, 205) and risk factor to early cancer related mortality (289, 290). The Tumour Node Metastasis (TNM) classification is currently the only established prognosis prediction tool and is used for most malignant tumours worldwide but has been criticised recently (1.7 Tumour node metastasis classification for oesophageal cancer). The accuracy and validity of the TNM classification for patients treated with neoadjuvant chemo(radio)therapy has been questioned as the classification was based on evidence from surgery alone treated patients (142-148). The finding in this study that both T and N category from the TNM (6^{th} edition classification) were independent

prognostic markers for cancer specific survival for patient not just from the surgery alone treatment group but also from the neoadjuvant chemotherapy followed by surgery treatment group, would suggests these concerns are unnecessary. This study results would support the findings of other recent studies which have all supported the prognostic importance of T and N category for OeC in both the 6th and 7th editions of the TNM classification (148, 292, 293).

Blood vessel and lymphatic involvement are not routinely assessed and were not part of the pathology minimum dataset at the time of the OE02 trial so this data was collected locally on receipt of resection specimens to LICAP. Using this local review data blood vessel invasion is shown to be an independent prognostic marker in the chemotherapy followed by surgery treatment group, while lymphatic invasion has been shown to be an independent prognostic marker, only in the surgery alone treatment group (for both overall and cancer specific survival). Both show prognostic value on univariate analysis in both treatment groups. It's often difficult to differentiate between lymphatic and vascular invasion and hence studies often report both together as "lymph-vascular" invasion (204, 294). The prognostic value of vascular invasion (blood vessel involvement) has been reported in prostate (295), breast (296) and colorectal cancers (297). In OeC there has been extensive literature showing the prognostic value of vascular involvement (175, 204, 294, 298-302) with evidence of blood vessel invasion at the tumour being associated with a poor prognosis. In this study we have shown an influence of the presence of vascular invasion on both overall and cancer survival but only in the chemotherapy followed by surgery treatment group is it was an independent prognostic marker. This has been shown in previous studies (204, 209, 303, 304), however, other studies have only shown blood vessel invasion to have prognostic value without being an independent prognostic marker (175, 299-302). Oesophageal cancer metastasis is more common via haematological routes than

locoregional recurrence (304, 305) so the presence of blood vessel involvement is likely to increase this possibility of this route of recurrence. However no recurrence data was available to assess for any patterns associated with those patients with evidence of blood vessel involvement. Blood vessel invasion clearly has prognostic importance but why it is only an independent prognostic marker in the neoadjuvant chemotherapy treatment group is unclear.

Lymphatic invasion is thought to be directly linked to the lymph node metastasis due to the creation of small lymphatic capillaries at the tumour microenvironment (306-308). In oesophageal cancer Osugi et al showed that lymphatic vessel invasion correlated with lymph node metastasis ($p<0.001$) in a cohort of squamous cell cancer patients treated with surgery alone (298). This they hypotheses was the reason that vascular or lymphatic invasion lead to poor outcomes by being a marker for distal micro-metastasis. Langer et al showed that lymphatic invasion was also significantly associated with survival ($p<0.001$) but also that the absence of lymphatic invasion correlated with tumour regression ($p<0.001$) (175). The findings in this cohort support these previous findings that the presence of lymphatic invasion in the surgery alone treatment group is an independent prognostic marker for both overall and cancer specific survival ($p=0.013$ and 0.003 respectively).

The survival benefit between the two treatment groups identified in the OE02 trial publications is not shown to be significant in the cohort of cases received by LICAP. The reasons for this are likely to be related to the 120 cases where no resection material was available in the non-received cohort (40.8%). In the initial OE02 published data, 4.5% of patients did not proceed to have any surgery in the trial because they died before surgery or where found to have unresectable tumour in the work up to surgery (i.e. disease progression) (99). There were also cases were the resection was not possible intra-operatively so were unlikely to have resection specimens (10%).

These cases with no resection material were likely to represent tumours with aggressive features and made up 14% of the surgery alone patients included in the original OE02 analysis. This may help to account for the improved survival seen in both treatment groups for the patients received by LICAP (Figure 13 and Figure 14).

4. QUANTITATIVE MEASUREMENT OF TUMOUR REGRESSION BY MEASURING TUMOUR CELL DENSITY

4.1 Background

Following the results from OE02 trial the “gold” standard treatment for locally advanced resectable OeC in the UK involves neoadjuvant cytotoxic combination chemotherapy followed by potential curative surgery (99). In the UK, the decision how a particular patient with oesophageal cancer is treated is made within a multidisciplinary team meeting considering results from endoscopy, pathology, imaging and patient’s individual performance status, age etc. A study in 1999 identified that patients with oesophageal cancer who respond to chemotherapy have a better survival than patients who underwent chemotherapy and did not respond (309). This finding was supported by other studies which all demonstrated that patients with oesophageal cancer who undergo neoadjuvant therapy, and show a complete pathological response have significantly better survival rates as high as 60% survival at 5 years (125, 185, 310, 311). Most importantly, patients who do not respond to neoadjuvant chemotherapy can have a worse outcome than if they had proceeded straight to surgery especially if there is tumour progression (112). As reported in the OE02 trial, chemotherapy can cause a variety of chemotoxic side effects such as vomiting, hair loss, severe fatigue, bronchial pneumonia and chemotherapy itself may lead to death. There was a 3% rate of deaths before surgery in the chemotherapy + surgery treatment arm (94). Neoadjuvant chemotherapy is time consuming as it usually requires at least 2 cycles and cycles are given over 2-4 week with breaks in between, all of which delay the time to potential curative surgery. Additionally, there might be significant distress and anxiety for the

patients during the chemotherapy which must be managed carefully throughout the process. At this moment in time, there is no tool available in clinical routine to identify the patients who will or will not benefit from neoadjuvant chemotherapy. Currently, the reaction of the tumour to chemotherapy (progression vs stable disease vs. regression) during treatment is assessed by radiological imaging. The World Health Organisation (WHO) introduced guidelines to ensure standardised reporting of the degree of radiological response to chemo(radio)therapy in 1981 (312). Since 2000, a simplified version of these guidelines known as the Response Evaluation Criteria in Solid Tumours (RECIST) has been used worldwide (313). The guidelines were updated and a new version (RECIST V1.1) was published in 2009 (314). RECIST uses an one-dimensional measurement (longest tumour diameter) instead of bi-dimensional measurements as previously recommended in the WHO guidelines (312) in an attempt to standardise the technique of assessing tumour response after neoadjuvant chemo(radio)therapy (314, 315). In patients with OeC, CT imaging is currently used to assess tumour regression using the RECIST criteria despite the multi-modality imaging used for the initial diagnosis and staging (see 1.8 Radiological prognostic markers in). At baseline i.e. before any neoadjuvant treatment commences, tumours are defined as either measurable (e.g. longest tumour diameter >10mm) or non-measurable (e.g. longest tumour diameter <10mm), while the criteria used for the assessment of lymph nodes uses the cut-off of 15mm to determine nodes suitable for assessment (314). Following neoadjuvant treatment repeat CT imaging is performed using the same one-dimensional measurements, enabling the primary tumour and lymph nodes to be classified as;

- Complete response (CR): Disappearance of all measurable primary tumour and all lymph nodes (previously >15mm) now reduced on their longest diameter <10mm

- Partial response (PR): 30% or more decrease in the diameter of the primary tumour compared to the pre-treatment diameters

- Progressive disease (PD): 20% or more increase in the diameters of target lesions using the smallest diameters measured

- Stable disease (SD): Neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease

Although the assessment of the longest tumour diameter has been shown to be an acceptable measure of response to chemotherapy (316), there is little evidence to show that response evaluation according to RECIST criteria can predict OeC patient's prognosis where the tumour margins can often be difficult to define after neoadjuvant chemotherapy (317). Another criticism of the use of the RECIST for evaluation of response to chemotherapy in OeC patients is related to the fact that at least some of the OeC might regress unevenly (190). This phenomenon has been referred to as post treatment 'centrifuge appearance' and makes reproducible identification as well as measurement of the longest diameter of the tumour difficult on CT images (121). This uneven regression changes may lead to an inaccurate radiological assessment of response and pathological stage (318-322).

Endoscopic ultrasound (EUS) is currently not accepted as a measurement tool for tumour response. The use of positron emission tomography (PET) in conjunction with CT for evaluation of tumour response is currently been considered (314). Several studies have shown the potential benefit of using PET imaging for assessment of tumour response during neoadjuvant therapy (316-321). In this context, the MUNCION (Metabolic response evalUatioN for Individualisation of neoadjuvant Chemotherapy In oesOphageal and oesophagogastric adeNocarcinoma) trial investigated prospectively the use of PET imaging to guide duration of neoadjuvant chemotherapy given to OeC

patients (320). Within that trial, baseline PET imaging was done before and 2 weeks after patients had commenced chemotherapy. Patients with evidence of response at 2 weeks continued to complete a 12 week course of chemotherapy, while 'non-responders' proceeded straight to surgery. After resection, histopathological response was assessed using Becker's tumour regression grading (178), which classified 8 (16%) patients from the PET identified 'responders' group as complete pathological response and 29 (58%) patients as major pathological response. None of the patients in the PET 'non-responder' group showed evidence of a major pathological response (320). Whilst these results were promising, validation of the results within a randomised trial is still awaited.

In order to individualise patient treatment, there is a clinical need for a reliable tumour regression grading system that could be used while the patient is still receiving treatment as well as after treatment. Such a tool could assist to potentially stop ineffective treatment therefore allow switching to other treatment/treatment modalities or indicate continuation of the current treatment.

The use of T-category (depth of invasion into oesophageal wall) of the tumour has been advocated to assess response/'downstaging' following neoadjuvant treatment (280). In this context, the initial radiologically determined T category (cT) using CT or EUS was compared to the pathologically determined pT category of the resection specimen (280). However, there is evidence that radiological T staging is not very accurate (322) and there is growing evidence that the T-category after neoadjuvant chemotherapy may not reflect pathological response (121, 147, 148, 180, 185). This is mostly related to the fact that the pT category is determined based on the deepest location of the primary tumour in the wall disregarding the amount of tumour present in the respective location or whether the tumour is continuously extending from the luminal surface to the deepest point or not. Thus, for example, if there are only 10

viable tumour cells left after neoadjuvant chemotherapy and these are located in the peri-oesophageal fat, the tumour is staged as ypT3 despite a major histopathological regression. Schneider et al demonstrated that from 19 OeC patients with a major pathological response after neoadjuvant chemotherapy only one tumour was actually "downstaged" with respect to its radiological T category (i.e. lower ypT category than T category predicted from radiological assessment). Guo et al reported similar findings and concluded that tumour regression grading (TRG) would better reflect the changes in the tumour due to neoadjuvant chemotherapy than the T category (121).

However, as outlined in section 1.9 Tumour regression systems used to assess oesophageal cancer), various different tumour regression grading systems exist worldwide with no consensus on which one should be used (177, 178, 323). Most TRG systems are criticised for being subjective with high levels of both, inter and intra-observer variability (174, 192, 196, 197).

Current TRG systems such as the Mandard (177), Becker (178) and Chirieac (194) classifications as well as the TNM classification (324) rely on the assessment of the resected specimen. A new system which potentially could monitor tumour response accurately while the patient is receiving chemotherapy could enable changes in patient management during chemotherapy treatment. This would ultimately lead to a personalised treatment approach with better patient outcomes and less unnecessary side effects. The use of endoscopic serial biopsies during neoadjuvant chemotherapy could be a possible option to evaluate TRG while the patient is still on treatment.

Only 1 previous study to date has attempted to quantify the amount of residual tumour in OeC. This study involved squamous cell carcinoma patients (n=183) who had undergone neoadjuvant chemoradiotherapy followed by curative surgery (180). They calculated tumour percentage from measurements of the residual tumour area and dividing this by the total area of the estimated previous tumour site. Patients were then

stratified into one of four pre-defined groups depending on their tumour percentage. The results showed that the percentage of viable tumour was an independent prognostic marker (p=0.005 in multivariate analysis) with patients with low residual tumour percentage having significantly better prognosis. Although well designed this study included a selected type of histological type of OeC and can be criticised for the subjective nature of determining the residual tumour cell percentage. The study also including no “control” group of patients who had not undergone any neoadjuvant treatment to compare tumour percentages so cannot determine whether some of the changes thought to be a result of scarring following neoadjuvant chemoradiotherapy may have occurred spontaneously. Two previous studies have looked at the stroma to tumour ratios in OeC, but both excluded patients who had undergone neoadjuvant chemo(radio)therapy (325, 326). Both studies used clinical estimates of the residual tumour through visualisation by 2 pathologists before assigning cases to either the high stroma group (>50% stroma) and low stroma group (<50% stroma). Both techniques do not improve on the criticism of existing regression grading systems in that they both involve a degree of subjective assessment. They both also placed patients into “ready-made” prognostic groups based on previously defined regression groups by Becker and Mandard (177, 178). Neither technique was able to produce quantified measures of tumour to stroma ratios allowing a continuous variable to be created and assessed for prognostic value.

Morphometric pathological measurements of different cell components was initially described by Chalkley in the 1940s (327, 328) and developed further by Weibel et al (329, 330). Morphometric measurements can provide a quantitative measurement of tumour cell density replacing subjective estimates. Quantitative measurement of tumour cell density (TCD) using morphometric point counting techniques have been used successfully in the past and most recently in colorectal and prostate cancer

studies taking advantage of digitised slides (198, 199, 331). Point counting is a morphometric technique that allows histological components to be measured objectively either using a microscope or nowadays using virtual (digitalised) slides (198, 199). This type of measurement will provide continuous data which can be used to identify the optimal cut off for prognosis prediction as opposed to predefined estimates such as used by Tong et al (cohort split into thirds) and Mesker et al (cohort split at 50%) (180, 325). Nowadays morphometric measurements are performed using digital slides ("virtual slides"). Virtual slides from a networked server have the advantage that they can easily be viewed by observers from any internet connected computer, facilitating collaborations and counterscoring by several observers. Also virtual slides can be annotated without affecting the original slide as annotations are saved separately.

The aim of this study was to quantify the tumour cell density (TCD) at the luminal surface of the tumour using a morphometric methodology in Haematoxylin/Eosin (H&E) stained slides from the resections specimens of patients recruited into the OE02 trial. Results will be compared between patients who were treated with neoadjuvant chemotherapy followed by surgery and those who were treated by surgery alone. The relationship with clinicopathological data including tumour regression grading according to Mandard and patient survival will be assessed.

4.2 Methods

4.2.1 Patients and material

H&E stained slides from 508 resection specimens from patients recruited into the OE02 trial (3.2.1 OE02 material local collection) were used for this study. Resection specimens (n=39) without primary tumour at the luminal surface or with no evidence of tumour in any of the material received were excluded from the measurements and further analysis. Patients with complete pathological response (n=11) in the resection

specimen according to the original pathology report were confirmed on local pathology review before being classified as TCD=0 and included in the analyses. All H&E stained slides from all resection specimens were scanned at x40 magnification using the Aperio XT slide scanner (Leica Biosystems, Nussloch, Germany) to create digital slides from each OE02 case. Images were stored on a secure server with internet access. No demographic or clinicopathological data was stored along with these files.

4.2.2 Establishing the shape of the measurement area.

A pilot study was performed to test the feasibility of measuring TCD at the luminal surface of the tumour using either a squared or circle shaped areas. Regardless of the shape the area the size was set as 9 mm^2 following previous work in the department (198). Both the size of the area and the luminal surface positioning were chosen to simulate endoscopic biopsies.

This feasibility study was performed in 20 randomly selected cases. The number of measurement points was 300 +/- 30 points for both approaches as previously established (198). Scoring categories and calculation of TCD are described below.

4.2.3 Tumour cell density (TCD) measurement

Digital slides were viewed using the Aperio ImageScope® (Leica Biosystems, Nussloch, Germany) programme blinded to any clinicopathological findings including treatment arm. The following criteria were used to ensure consistent slide and area selection:

STEP1: Selection of slide for scoring:

I. All available slides of an individual case were reviewed to identify all slides with viable primary tumour.
II. Slides which contained viable tumour without tumour at the luminal surface were excluded.

III. From the slides with viable tumour at the luminal surface, the slide with the deepest infiltration of tumour in the wall was chosen for TCD measurement.

IV. In the event that more than one slide had tumour at the luminal surface with the same depth of invasion, the slide with the highest estimated density of tumour was selected.

STEP 2: Selection of the 'measurement area' within the slide:

I. the area of tumour which appears to have the highest viable tumour cell density at the luminal surface on 'eyeballing' was selected

II. A 9mm^2 area was created using the pen-tool of the software and superimposed onto the virtual slide. The observer positioned the area in a way that the measurement area had maximal contact with the luminal surface and contained tumour throughout.

Once this superimposed area was determined, the RandomSpot® (University of Leeds, Leeds, UK) software (199) was used to generate measurement points throughout the 9mm^2 area. The software will create the first measurement point at a random position and then all further points equidistant to each other (199). This process is known as 'systematic random sampling' and has been shown to be an effective and reproducible technique (199, 329, 332). For this study, we used 300 points per area +/- 10%. The number of measurement points needed to provide an accurate estimate of the volume fractions depends on the number of events (333) e.g. the lower the event, the more measurement points are needed. Studies in the past including our own (198) have shown that for the accurate measurement of the proportions of tumour and stroma, 300 +/- 10% measurement points per 9mm^2 area provide a satisfactory result.

Every individual measurement point was viewed by the observer at 20x to 40x magnification and a category was assigned to each measurement point, adapted from work done previously in the department (198):

0= Non informative (normal tissue or empty area or inconclusive)

1= Tumour

2= Stroma including fibrosis

3= Necrosis

4= Blood or lymphatic vessel

5= Inflammatory cells irrespective of type

6= Tumour lumen (regardless of content)

7= Mucin (not in tumour lumen)

8= Muscle

In situations where the measurement point was found on an area of empty space such as when there is a tear in the slide material the point was allocated depending on the surrounding cells. Therefore if the point is found in an area of tumour with tour cells either side then the area with the tear would be taken as also a tumour point. In the event where it was different tissue/cells around the indeterminate scoring point, so that an assumption couldn't be made, this point would be scored "0" for non informative. The category of each point was entered individually into the annotation box of the Aperio Imagescope software and all values per case were saved as XML file and excel spreadsheet to enable statistical analyses. The categories 'tumour (category 1)' and 'tumour lumen (category 6)' were combined as 'tumour' for analyses and compared to 'stroma' for which stroma/fibrosis (category 2), necrosis (category 3), vessels (category 4), inflammatory cells (category 5), Mucin (category 7) and muscle (category 8) were combined. Non informative points were not included in the analyses.

The tumour cell density was calculated and expressed as a percentage of the total number of informative points. The total informative points were taken as the sum of all points labelled minus the points labelled “0” for a non-informative.

$$\textbf{\textit{Tumour cell density}} = \left({}^{(number\ of\ 'tumour'\ points)} \Big/ _{(total\ informative\ points)} \right) \times 100$$

The observer (TS) was trained in categorisation of the points by an experienced gastrointestinal pathologist (HG) and performed all scoring blinded to any clinicopathological data. The observer was also not aware of the treatment modality.

4.3 Intra- and inter-observer studies

As this study involved potentially subjective slide selection rule and rules for position of the measurement area, intra-observer variability of performing these tasks was evaluated. Furthermore, inter-observer agreement was assessed for the categorisation of the measurement points.

4.3.1 Slide Selection

To assess intra-observer variability of the initial slide selection, 40 cases were re-reviewed and the most suitable slide for analysis was re-selected by observer 1 (TS). This was done after the observer had initially reviewed 200 cases and repeated again after the observer had completed reviewing all cases (n=508).

4.3.2 Measurement area selection

To assess intra-observer variability of the placement of the measurement area, 40 cases were reassessed and a measurement area created by observer 1 (TS). This test was again done after observer 1 had reviewed and point counted 200 cases. The intra-observer agreement was considered satisfactory if the area was positioned with a >50% overlap of the originally measurement area.

4.3.3 Tumour cell density measurement

1. To assess for intra-observer variation in assigning the different categories to the measurement points, observer 1 (TS) re-assigned categories in 20 randomly selected cases. To investigate whether there is a learning curve of the categorisation capability of the observer, 20 cases were re-assigned after the first 200 cases had been scored and then re-assigned for a third time at the end of the study. Kappa agreement was determined with a kappa greater than 0.75 defined as excellent, 0.45-0.74 defined as good and <0.40 defined as barely reproducible.

2. To assess for inter-observer variation a trained histopathologist (Gordon Hutchins, GH) re-categorised 50 randomly selected cases using the original measurement grid blinded to the categories of the first observer. The same categorisation rules were applied. TCD values of these 50 cases were compared between observer 1 (TS) and observer 2 (GH).

The agreement per individual measurement point and end result e.g. TCD were compared. Bland Altman analysis (334) and Kappa statistics were performed.

4.4. Statistical analyses

Statistical analysis was performed using the computer program Statistical Package for the Social Sciences (SPSS) 21.0 (Chicago, Illinois).Differences between intra-observer TCD scores will be assessed and a mean of these differences produced. Intra- and inter-observer agreement is tested using a kappa agreement measure. A kappa value equal or greater than 0.70 was deemed satisfactory. Bland Altman plots were constructed to compare TCD scoring between the 2 observers.

The relationship between clinicopathological features (age, gender, histology type, grade of differentiation, T category, N category, blood vessel invasion, lymphatic vessel invasion and Mandard tumour regression grading) and TCD were analysed for the

whole group and then by individual treatment groups using Kruskal-Wallis or Mann-Whitney test.

In order to identify the optimal TCD cut off for Kaplan Meier survival plots and see whether there is a linear relationship between TCD and survival, survival plots from 5, 4, 3 and 2 equally sized groups were created. Kaplan Meier survival plots were visually evaluated to decide which groups might best be combined and establish the final cut off point.

Survival data was calculated from time of randomisation to last follow up or death using the Kaplan-Meier method (270) and differences between groups were tested by the log-rank test. All survival analyses were done separately by treatment arm.

For cox regression univariate survival analysis the relationship between survival and age, gender, histology type, tumour differentiation, pT category (TNM6), pN category (TNM6), evidence of lymphatic invasion, blood vessel invasion, Mandard grading and TCD (continuous variable) was assessed. For multivariate analysis, only variables with significant p values in univariate analysis were included in the model. A p-value of less than 0.05 was considered to be significant.

4.5 Results

4.5.1 Pilot work – shape of the measurement area

This technique was designed to use a standard area for TCD calculations, taken from the luminal surface hence give results that maybe comparable to TCD calculations from biopsy tissue. In 9 (60%) cases of this pilot study, the use of a square shaped area would only allow a corner of the measurement area to be kept in contact with the luminal surface (see Figure 15). This was at least partly related to the fact that the Aperio ImageScope® software (Leica Biosystems, Nussloch, Germany) did not allow the measurement area to be rotated.

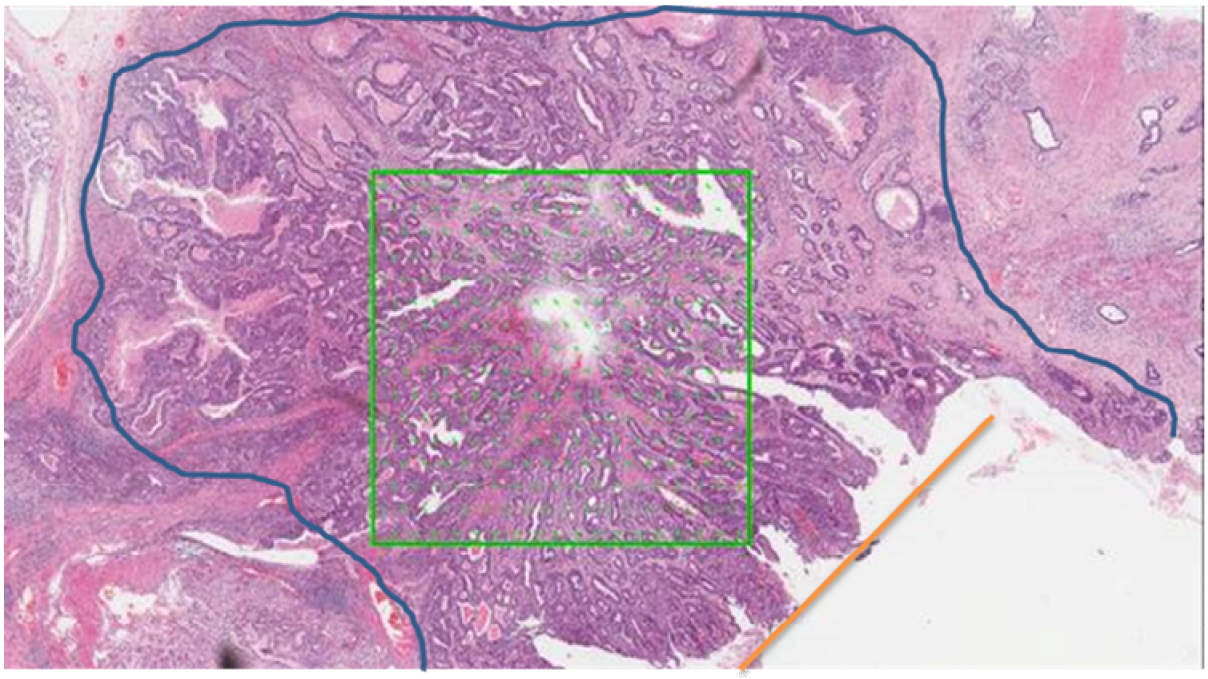


Figure 15: H&E stained digital slide of oesophageal adenocarcinoma with 9mm^2 square measurement area.
The H&E slide shows an area of oesophageal adenocarcinoma at the luminal surface outlined in blue. A square area (green box) for potential TCD measurement was placed in the tumour region, but only the bottom right corner of this area is actually in contact with the luminal surface (orange line). Green scoring points can be seen equidistantly distributed through the area.

A circular 9mm^2 measurement area was tried as an alternative to the square shaped area in the same 15 cases. In all cases, the contact of the circular area with the luminal surface was far greater than using the square shaped area (see Figure 16). After this initial pilot work, a decision was taken, that cases will be measured with a 9mm^2 circular area placed at the luminal surface.

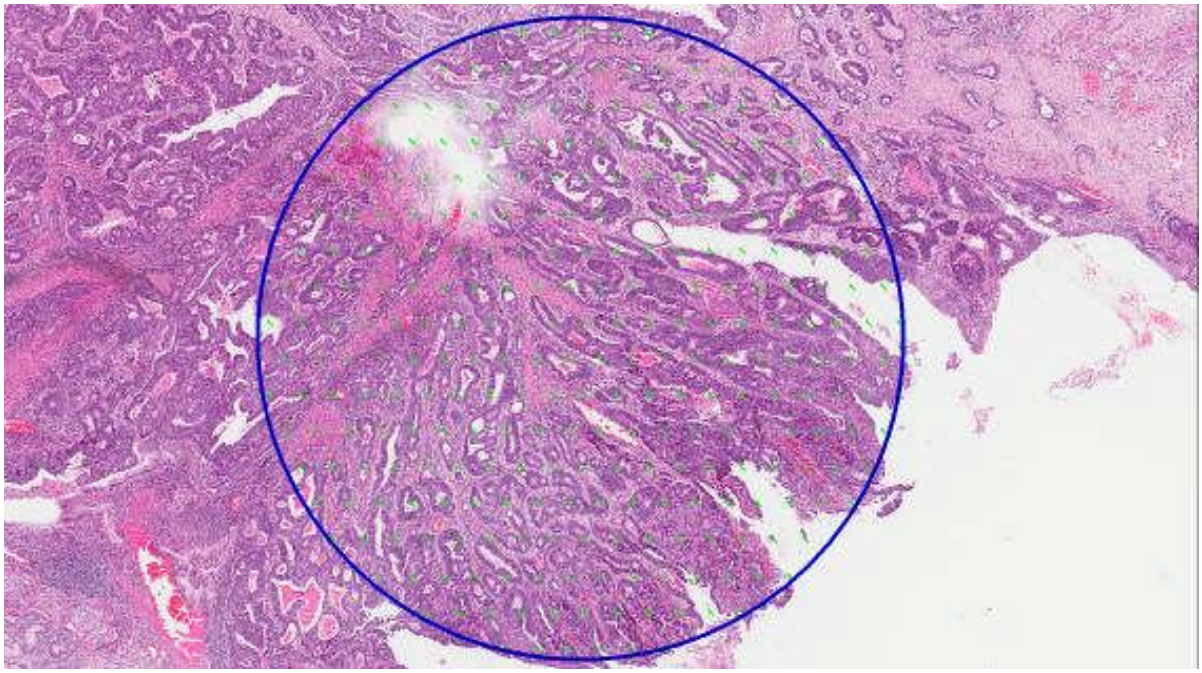


Figure 16: H&E stained digital slide of oesophageal adenocarcinoma with 9mm^2 circular measurement area.
The H&E slide shows an area of oesophageal adenocarcinoma at the luminal surface outlined in blue. A circle area (blue outline) for potential TCD measurement was placed in the tumour region with a good large area of contact with the luminal surface. Green scoring points can be seen equidistantly distributed through the area.

4.5.2 Intra-observer variation of slides selection and measurement area placement

4.5.2.1 Slide selection

Intra-observer variation of slide selection for measurement was done in 20 cases at two different time points: (1) after the first 200 cases and (2) at the end of the whole experiment (508 cases). At time point (1) in 15 of the 20 cases (75%), the same slide was selected. At time point (2), a further 20 cases were re-assessed and this time in 19 (95%) cases the same slide was selected. This data suggests that there was a learning curve of the observer. After discussion with the supervisor, it was decided that this represents an acceptable level of agreement.

4.5.2.2 Measurement area placement

Using the same 40 cases assessed for slide selection intra-observer variability but excluding the ones where there was an initial disagreement in slide selection (n=6), the

circular measurement area was re-selected using the same rules as for the initial placement (see 4.2.3 Tumour cell density (TCD) measurement). The placement of the 'original' measurement area and the 'reselected' measurement area were reviewed. The reselection was classified "acceptable" if the measurement area was placed in the exact same position or within 'close proximity' (at least >50% overlap between the two positons of the measurement areas).

In 29 cases (85%) the measurement area was re-positioned in an acceptable position of which in 20 cases (59%) the scoring area was in the identical position as the original position. The 5 cases that did not achieve good intra-observer agreement are summarised in Table 14. In 2 cases, there was some overlap in the position of the measurement areas, but the overlap was <50%. In 3 cases, there was no overlap of the circles at all and in all three cases, the TCD increased between 33% and 58% compared to the original circle.

Table 14: Tumour point frequencies and TCD % differences in the 5 cases with disagreement of scoring position.
Cases 1 and 2 had the measurement areas placed in close proximity but overlap was < 50%. Cases 3, 4 and 5 the measurement areas did not overlap at all.

Case number	1st measurement area position No. tumour points (TCD %)	2nd measurement area position No. tumour points (TCD %)	Difference in TCD%
Case 1	138 (48)	105 (37)	-9%
Case 2	116 (40)	92 (32)	-8%
Case 3	128 (46)	228 (79)	+33%
Case 4	50 (17)	216 (75)	+58%
Case 5	108 (38)	208 (72)	+34%

4.5.3 Intra-observer variation of categorisation of measurement points

20 cases were randomly selected and re-categorised blinded to the original or previous categorisation. Results are shown in Table 15. The average difference in TCD percentage between the original categorisation and the 1st rescore (performed after

200 cases had been scored) was 8.2%; between the 1st rescore and the 2nd rescore this average difference had reduced to 4.8%. The average difference between the original score and the 2nd rescore was higher at 13.5%. This reduction in TCD difference in later scoring suggests an improvement in scoring through the process with more consistent scoring.

Table 15: Intra-observer variability in categorisation of measurement points.

Case number	Original TCD (%)	1st TCD rescore (%)	2nd TCD rescore %	Kappa agreement Original vs. 1st	Kappa agreement 1st vs. 2nd	Kappa agreement Original vs. 2nd
UGI0006	51.2	41.8	45.6	0.78	0.79	0.79
UGI0041	74.7	71.9	82.3	0.44	0.61	0.55
.UGI0062	30.2	29.2	31.3	0.94	0.90	0.87
UGI0084	57.0	59.5	60.5	0.80	0.86	0.79
UGI0094	73.3	71.5	75.0	0.86	0.84	0.87
UGI0112	21.5	31.0	51.7	0.58	0.47	0.32
UGI0728	38.3	44.9	43.2	0.76	0.85	0.79
UGI0737	40.4	46.0	43.2	0.75	0.71	0.75
UGI0747	30.3	28.9	27.5	0.86	0.85	0.81
UGI0773	54.7	56.4	56.8	0.84	0.84	0.78
UGI0796	56.9	58.3	60.1	0.82	0.87	0.85
UGI0911	55.7	49.3	59.4	0.83	0.78	0.80
UGI0958	43.6	44.6	43.6	0.81	0.81	0.79
UGI0973	75.6	74.6	80.1	0.92	0.83	0.86
UGI1012	27.5	51.6	53.3	0.49	0.67	0.47
UGI1023	30.0	67.6	75.6	0.28	0.67	0.20
UGI1037	41.1	44.3	46.3	0.83	0.89	0.80
UGI1060	19.9	64.1	82.9	0.30	0.47	0.13
UGI1081	17.4	55.1	61.3	0.27	0.54	0.22

TCD= tumour cell density.

The median kappa agreement between the original TCD scoring and the 1st rescore was 0.8 with a range (0.27 to 0.90 agreement). The median agreement between the 1st rescore and the 2nd rescore shows even closer agreement with the median kappa agreement of 0.81 and a range of agreement between 0.47 and 0.90. The median agreement even between the original and the second rescore remained good 0.79 (range 0.13 to 0.87) despite the improved scoring correlation later in the process.

4.5.4 Inter-observer variability

4.5.4.1 Point by point agreement analysis

For the inter-observer variability assessment GH reviewed 50 randomly selected cases and 48 of them were found suitable for rescoring. Two cases were deemed inadequate to be scored accurately and were excluded from all analyses. In total categorisation of 13877 measurement points were compared between TS and GH. 3 errors due to incorrect typing of the category were found and excluded from assessment. The comparisons of the non-informative points, tumour and stroma point agreement between the two observers is summarised in Table 16.

Table 16: Inter-observer agreement in assigning categories.

	GH frequency	TS frequency	% agreement
0= 'Non informative'	326	165	50.6
1+6= 'Tumour'	6271	5563	88.7
2+3+4+5+7+8= 'Stroma'	7280	5464	75.0

This table also shows that GH identified a total of 6271 tumour points (label 1 or 6) and in 88.7% TS agreed. Kappa statistic estimate was performed to assess inter-observer agreement for individual points per case; Kappa agreement = 0.73 (95% CI = 0.728 – 0.737, $p<0.0001$).

4.5.4.2 Case by case TCD agreement

The TCD comparison between the two observers (TS and GH) showed the median TCD value in TS scoring was 53.0% TCD (range 16.4 to 98.8%) compared to GH whose median was lower at 46.0% (range 12.6% to 91.4%). The mean difference in TCD each case was 6.6% and standard deviation of +/- 7.61 (Appendix 3: Inter-observer TCD score comparison). Bland Altman plot shows good agreement with most (81.2%) score differences within 1 Standard deviation of the mean (Figure 17).

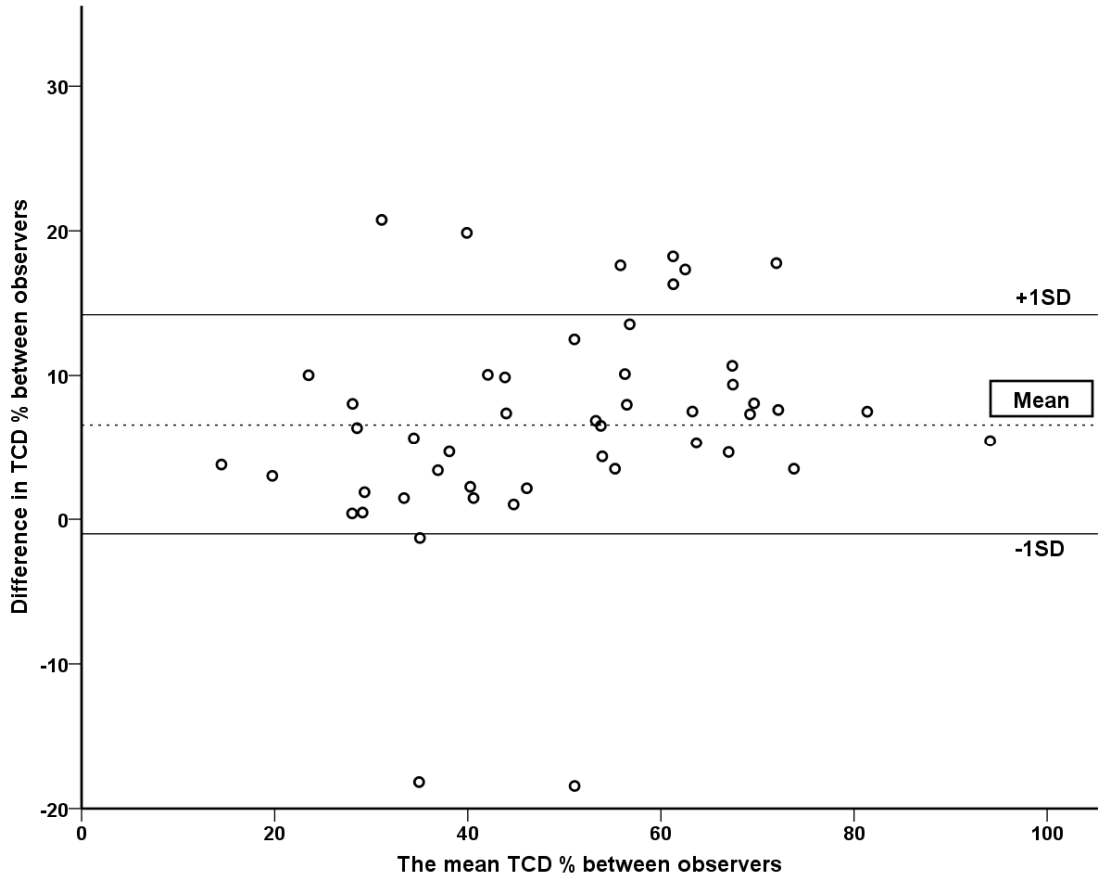


Figure 17: Bland Altman plot comparing the TCD scoring of two observers.
The bland Altman plot demonstrates the distribution of the 48 cases scored by two observers (TS and GH). Mean is at 6.6% with standard deviation lines either side by 7.61%.

4.6 Patient cohort

Slides or blocks from a total of 508 resection specimens from the OE02 trial were retrieved retrospectively (see Chapter 3. The OE02 thesis cohort's clinicopathological characteristics and survival). After applying the inclusion/exclusion criteria (see 4.2.1 Patients and material) slides from 469 patients were included in the final TCD analysis (Figure 18). Eleven cases with complete pathological response were classified as 0% TCD.

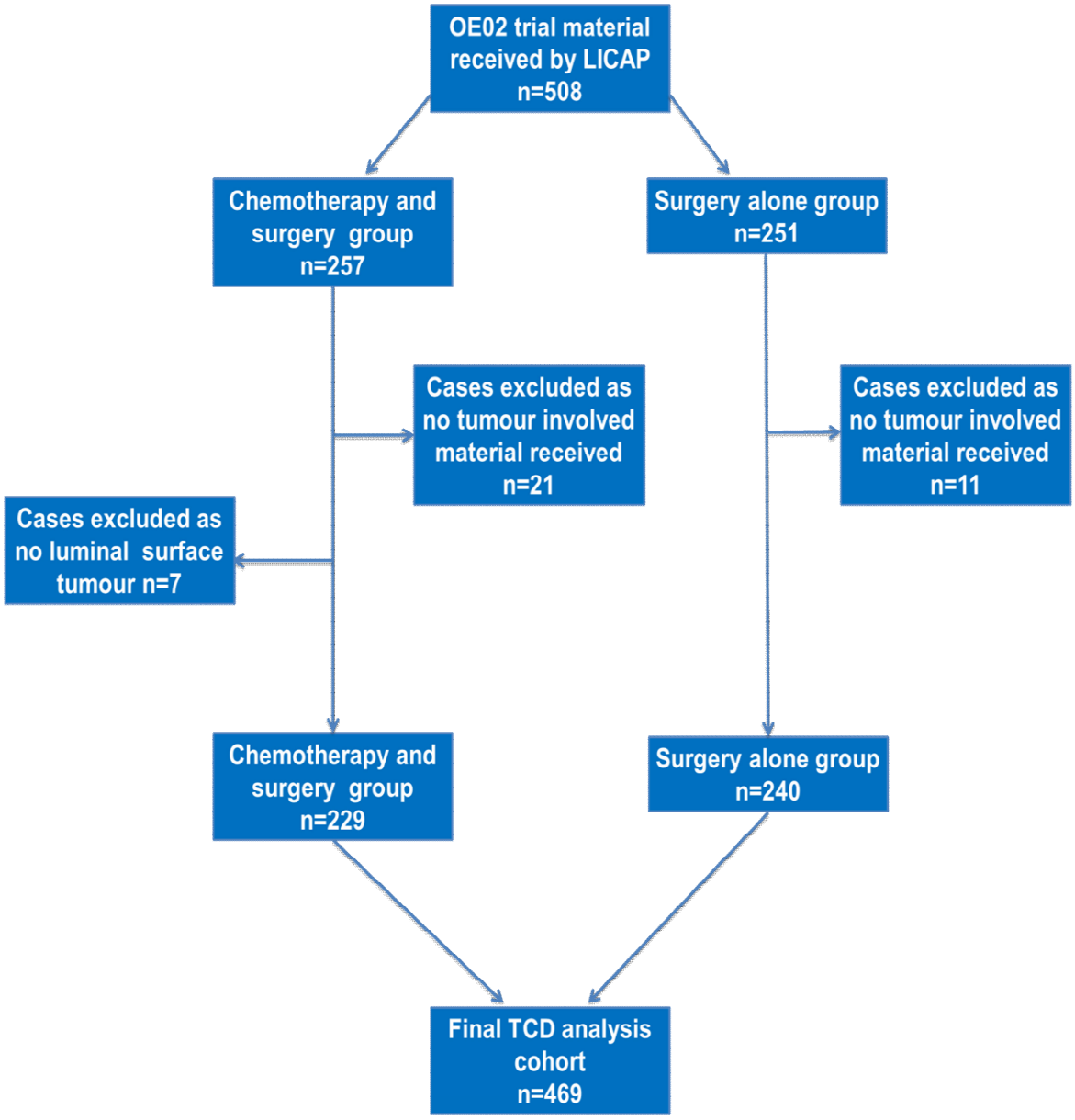


Figure 18: TCD final cohort creation flow chart.
CONSORT diagram illustrating the cases excluded from TCD analysis and the stepwise creation of the final cohort.

In the final cohort 358 (75.8%) patients were male and 229 (48.8%) were treated with neoadjuvant chemotherapy followed by resection (Table 17). The median (range) age was 62.9 years (30-83.1 years). In 11 cases there was no residual tumour in the resection specimens (per definition TCD =0%).Using the originally collected biopsy data revealed that 6 cases were squamous cell carcinomas, 4 adenocarcinomas and 1 case with other histology.

Table 17: Clinicopathological characteristics of the OE02 trial tumour cell density study cohort.

	Overall cohort n=469	
	n	%
Gender		
Male	358	76.3
Female	111	23.7
Treatment arm		
Chemotherapy+ surgery	229	48.8
Surgery alone	240	51.2
Histology		
Adenocarcinoma	331	70.6
Squamous	111	23.7
Other	16	3.4
No residual tumour	11	2.3
Grade of tumour differentiation		
Poor	204	43.5
Moderate	209	44.6
Well	41	8.7
Unknown	4	0.8
No residual tumour	11	2.3
(y)pT category±		
T0 (No residual tumour)	11	2.3
T1	40	8.5
T2	51	10.8
T3	355	75.4
T4	12	2.5
(y)pN category+		
N0	181	38.6
N1	288	61.4
Lymphatic vessel invasion		
Yes	160	34.1
No	270	57.6
Suspicious	37	7.9
Unknown	2	0.4
Blood vessel invasion		
Yes	59	12.6
No	374	79.7
Suspicious	35	7.5
Unknown	1	0.2
Mandard tumour regression grade*		
TRG1	11	2.3
TRG2	9	1.9
TRG3	37	7.9
TRG4	167	35.6
TRG5	244	52.0
Unknown	1	0.2

* Mandard grading; TRG 1= complete regression, TRG2= only a few residual tumour cells evident, TRG3= predominantly fibrosis, TRG4= predominantly tumour cells and TRG5= no evidence of any regression seen. ±TNM staging was performed according to the 6th edition of the UICC TNM staging manual (140)

Median (range) follow up time from randomisation was 1.49 years (0.02 to 13.21) and from surgery to last follow up or death 1.39 years (0.0 to 13.18). At the end of the follow up period 97 patients (20.7%) were still alive, 289 patients (61.6%) had died of cancer, 28 patients (6.0%) had died of complications related to their surgery and 48 patients (10.2%) had died due to factors unrelated to surgery or cancer. In 7 patients, the cause of death was unknown. Of the patients who were alive at the end of the study period, the median (range) follow up time from randomisation was 6.1 years in the chemotherapy followed by surgery treatment group and 5.8 years in the surgery alone treatment group.

The clinicopathological variables by treatment arm (surgery alone vs neoadjuvant chemotherapy plus surgery) are illustrated in Table 18. There was no significant difference between the treatment arms with respect to age, gender, histology, differentiation of the tumour and T category using TNM6 (Table 18). However, there was significant difference in the N category, lymphatic vessel invasion, blood vessel invasion and Mandard regression grading between the two treatment arms in this cohort. Patients treated by chemotherapy followed by surgery had a higher percentage of N0 disease, lower proportion of lymphatic vessel or blood vessel involvement and more frequent evidence of primary tumour regression (Table 18).

Table 18: Clinicopathological characteristics by treatment arm.

	Overall cohort n=469	Chemo+ surgery (n=229)	Surgery alone (n=240)	
	N (%)	N (%)	N (%)	P value
Age				
Median	62.7	62.5	63.5	0.423
Range	30.0-83.1	36.4-83.1	30.0-80.7	
Gender				
Male	358 (76.3)	182 (79.5)	176 (73.3)	0.118
Female	111 (23.7)	47 (20.5)	67 (26.7)	
Histology				
Adenocarcinoma	331 (70.6)	163 (71.2)	168 (70.0)	0.895
Squamous	111 (23.7)	51 (22.3)	60 (25.0)	
Other	16 (3.4)	7 (3.1)	9 (3.8)	
No residual tumour	11 (2.3)	8 (3.5)	3 (1.3)	
Grade of differentiation				
Poor	204 (43.5)	92 (40.2)	112 (46.7)	0.709
Moderate	209 (44.6)	109 (47.6)	100 (41.7)	
Well	41 (8.7)	18 (7.9)	23 (9.6)	
Unknown	4 (0.8)	2 (0.8)	2 (0.8)	
No residual	11 (2.3)	8 (3.5)	3 (1.3)	
(y)pT stage±				
T0 (No residual)	11 (2.3)	8 (3.5)	3 (1.3)	0.110
T1	40 (8.5)	19 (8.8)	21 (8.8)	
T2	51 (10.9)	29 (12.7)	22 (9.2)	
T3	355 (75.7)	169 (73.8)	186 (77.5)	
T4	12 (2.6)	4 (1.7)	8 (3.3)	
(y)pN category±				
N0	181 (38.6)	101 (44.1)	80 (33.3)	***0.017***
N1	288 (61.4)	128 (55.9)	160 (66.7)	
Lymphatic vessel invasion				
Yes	160 (34.1)	61 (26.6)	99 (41.3)	***0.006***
No	270 (57.6)	149 (65.1)	121 (50.4)	
Suspicious	37 (7.9)	19 (8.3)	18 (7.5)	
Unknown	2 (0.4)	0 (0.0)	2 (0.8)	
Blood vessel invasion				
Yes	59 (12.6)	16 (7.0)	43 (17.9)	***<0.001***
No	374 (79.7)	199 (86.9)	175 (72.9)	
Suspicious	35 (7.5)	14 (6.1)	21 (8.8)	
Unknown	1 (0.2)	0 (0.0)	1 (0.4)	
Mandard tumour regression grade*				
TRG1	11 (2.3)	8 (3.5)	3 (1.3)	***<0.001***
TRG2	9 (1.9)	7 (3.1)	2 (0.8)	
TRG3	37 (7.9)	26 (11.4)	11 (4.6)	
TRG4	167 (35.6)	93 (40.6)	74 (30.8)	
TRG5	244 (52.0)	95 (41.5)	149 (62.1)	
Unknown	1 (0.2)	0 (0.0)	1 (0.4)	

Mandard grading; TRG 1= complete regression, TRG2= only a few residual tumour cells evident, TRG3= predominantly fibrosis, TRG4= predominantly tumour cells and TRG5= no evidence of any regression seen. + TNM staging according to the 6th edition of the UICC TNM staging manual.

Next, was to establish the relationship between TCD and survival per treatment arm and determine the potential best cut off value for stratifying patients into groups with different prognosis.

4.7 Tumour cell density results

4.7.1 TCD % distributions and their relationship to the treatment groups

A total of 135054 points were categorised of which 132607 were deemed ‘informative’ (dropout rate: 1.8%). The median number of points reviewed per case was 287.0 (range: 270 to 320 points) with a median number of informative points of 282.8 (range: 206 to 303 points). The median number of tumour points per case (Figure 19) was 145.7 points (range: 0 to 273) and the median TCD percentage (Figure 20) was 53.2% with a range from 0 to 95.5% TCD.

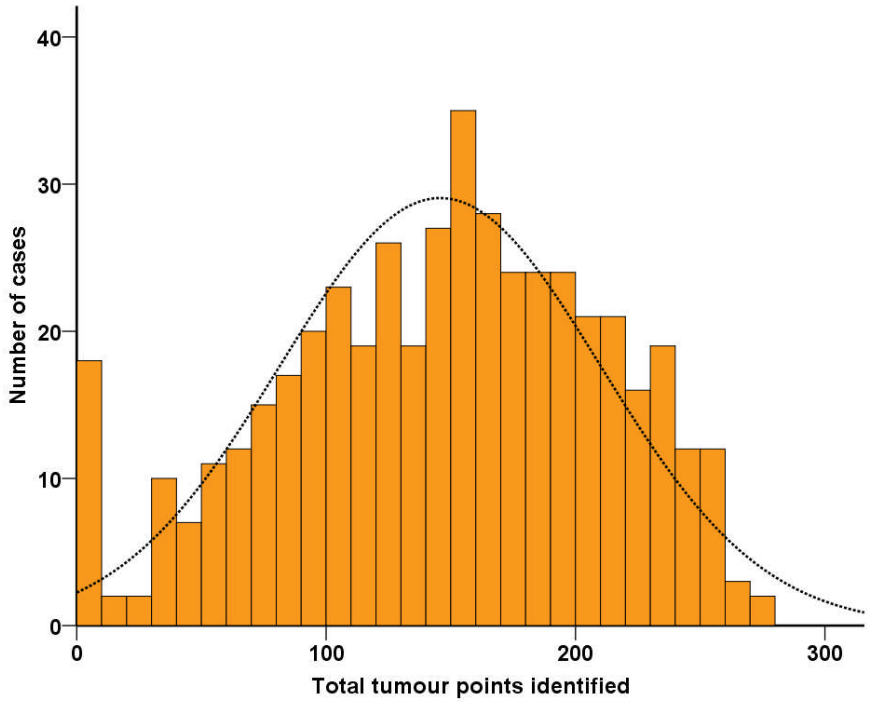


Figure 19: Histogram of tumour points per case in the whole TCD cohort
Histogram showing that the distribution of the number of tumour points per case follows a normal distribution

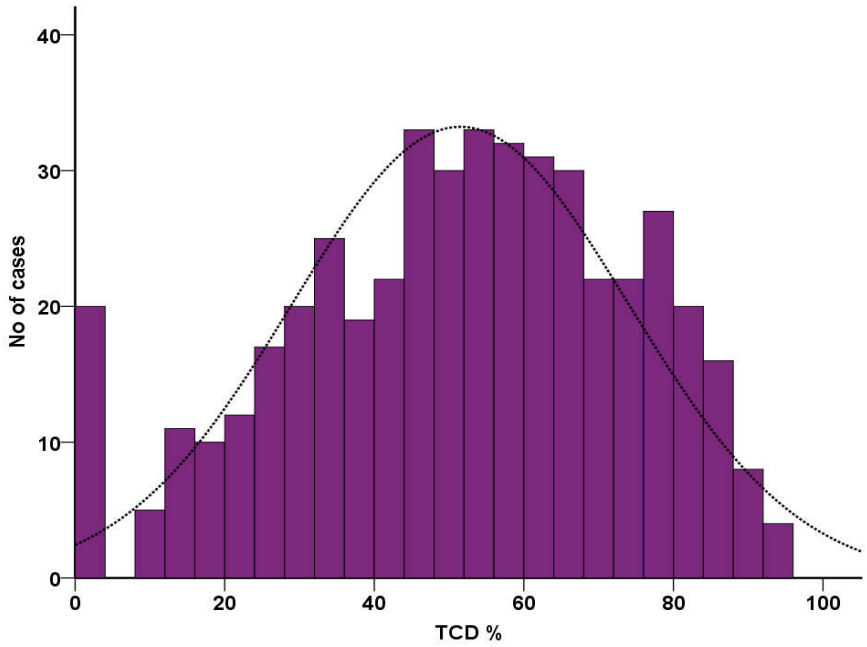


Figure 20: Histogram showing TCD % per case in the whole TCD cohort
Histogram showing that the distribution of the TCD % per case follows a normal distribution

There was a significant difference in TCD % between the two treatment arms (p<0.001). The median TCD in the chemotherapy + surgery group was significantly lower compared to the median TCD in the surgery alone group (median (range) TCD chemotherapy and surgery arm: 50.0% (0-93.1%), surgery alone arm: 55.6% (0-95.5%), p<0.001).

4.7.2 Identification of the cut off for prognostic stratification

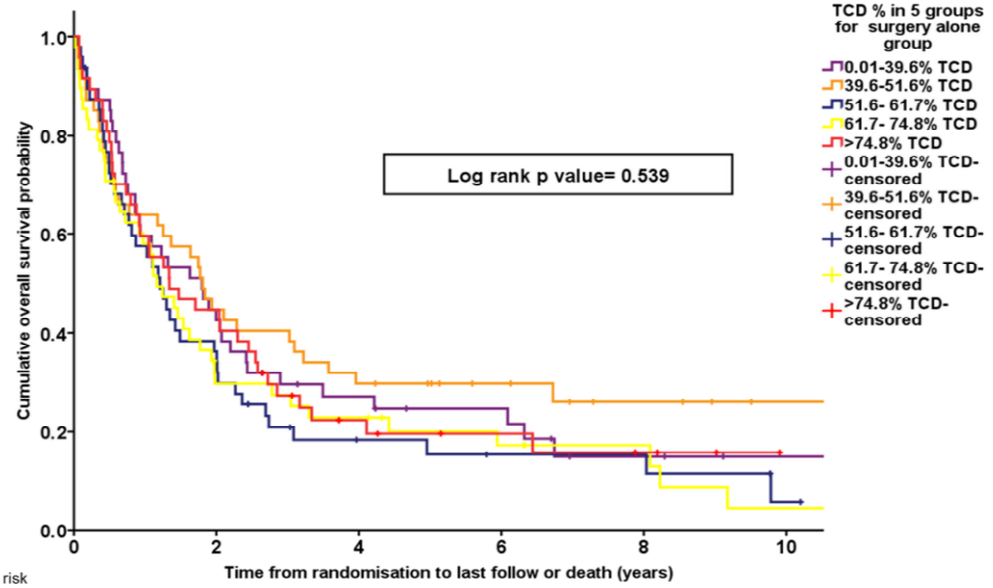
Equal sized TCD groups were created as set out in the methods (4.2 Methods). The cut-offs and the respective group sizes are shown in the Table 19 below:

Table 19: TCD range by group classifications and by treatment arm

	Chemotherapy and surgery group (n=229)		Surgery alone group (n=240)	
	TCD range %	No. cases	TCD range %	No. cases
Complete responders (T0)				
	0.0	8	0.0	3
5 equally sized groups				
1st fifth	0.0 to 28.4	44	0.0 to 39.6	47
2nd fifth	>28.4 to 44.5	44	39.6 to 51.6	47
3rd fifth	>44.5 to 51.2	45	51.6 to 61.7	48
4th fifth	>51.2 to 70.8	44	61.7 to 74.8	48
5th fifth	>70.8 to 100	44	74.8 to 100	47
4 equally sized groups				
1st quartile	0.0 to 31.6	55	0.0 to 43.6	59
2nd quartile	>31.6 to 50.7	55	43.6 to 55.6	59
3rd quartile	>50.7 to 67.4	56	55.6 to 71.1	60
4th quartile	>67.4 to 100	55	71.1 to 100	59
3 equally sized groups				
Low third	0.0 to 37.1	74	0.0 to 47.4	79
Mid- third	>37.1 to 61.0	73	47.4 to 65.4	79
High- third	>61.0 to 100	74	65.4 to 100	79
2 equally sized groups				
Low group	0.0 to 50.7	111	0.0 to 55.6	118
High group	>50.7 to 100	110	55.6 to 100	119

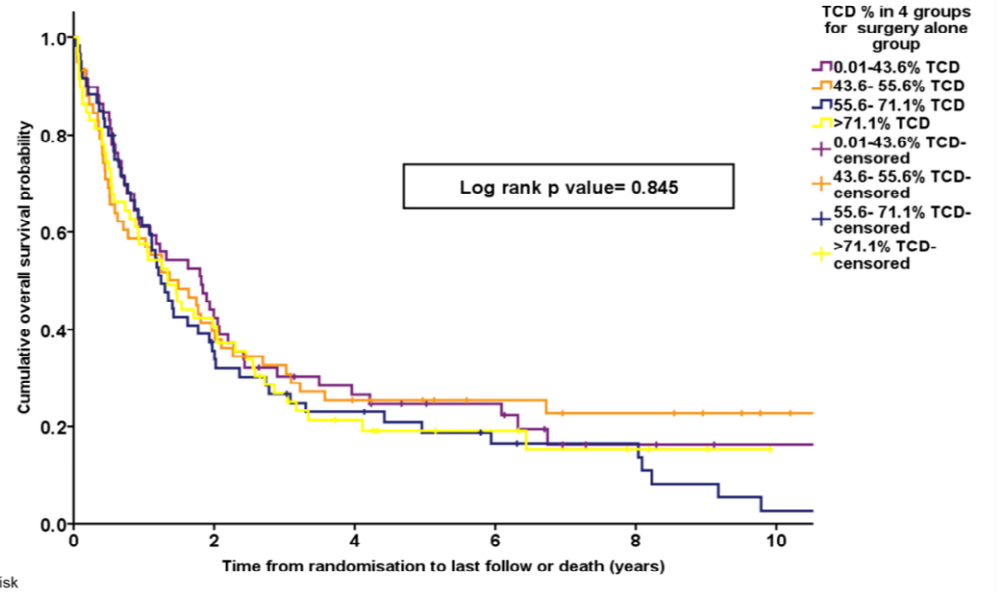
Using this patient stratification, Kaplan Meier survival plots were created for both overall and cancer specific survival using log rank test for assessing the statistical significance. None of the groupings showed as significant relationship between TCD and survival in the surgery alone group (all p-values > 0.05) (Figure 21 and Figure 22).

a.)

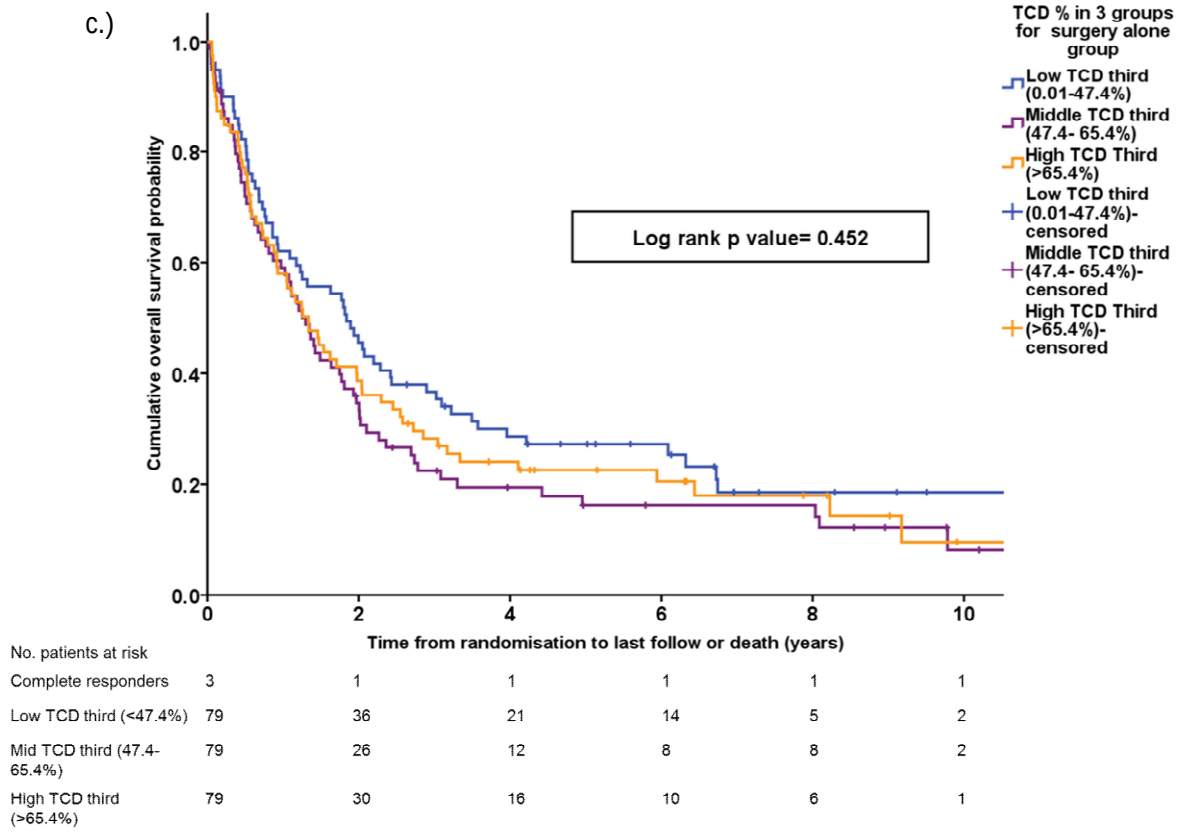


No. patients at risk						
Complete responders	3	1	1	1	1	1
0-39.6% TCD	47	20	11	8	3	1
39.6- 51.6% TCD	47	21	14	9	5	2
51.6-61.7% TCD	48	17	6	4	4	1
61.7- 74.8% TCD	48	13	10	6	4	1
>74.8% TCD	47	21	8	5	3	0

b.)

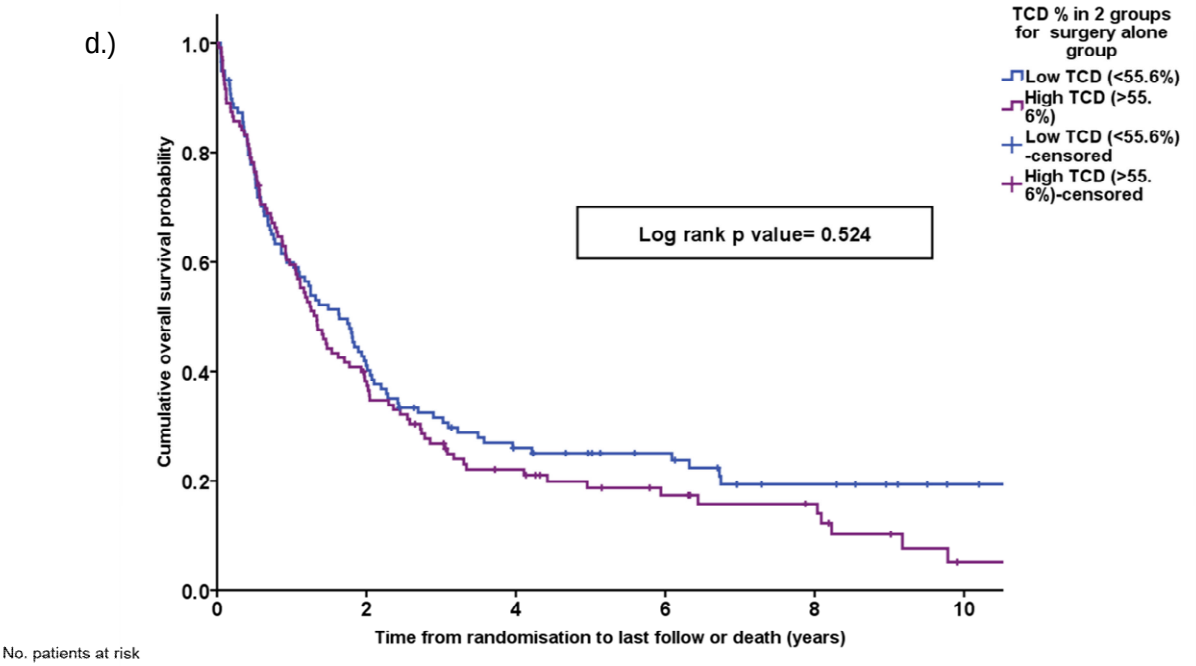


No. patients at risk						
Complete responders	3	1	1	1	1	1
1st quartile group	59	25	14	10	3	1
2nd quartile group	59	23	13	9	7	3
3rd quartile group	60	20	12	7	6	1
4th quartile group	59	24	10	6	3	0



No. patients at risk

Complete responders	3	1	1	1	1	1
Low TCD third (<47.4%)	79	36	21	14	5	2
Mid TCD third (47.4-65.4%)	79	26	12	8	8	2
High TCD third (>65.4%)	79	30	16	10	6	1



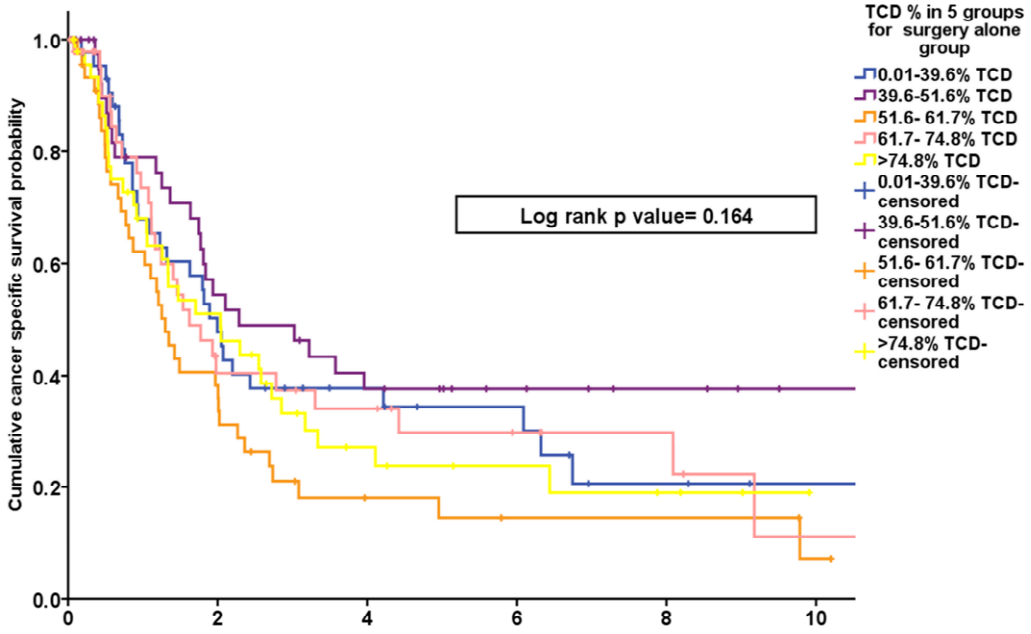
No. patients at risk

Complete responders	3	1	1	1	1	1
Low TCD (<55.6%) group	118	48	27	19	10	4
High TCD (>55.6%) group	119	44	22	13	9	1

Figure 21(a-d): Univariate overall survival analysis by TCD grouping in the surgery alone treatment group.

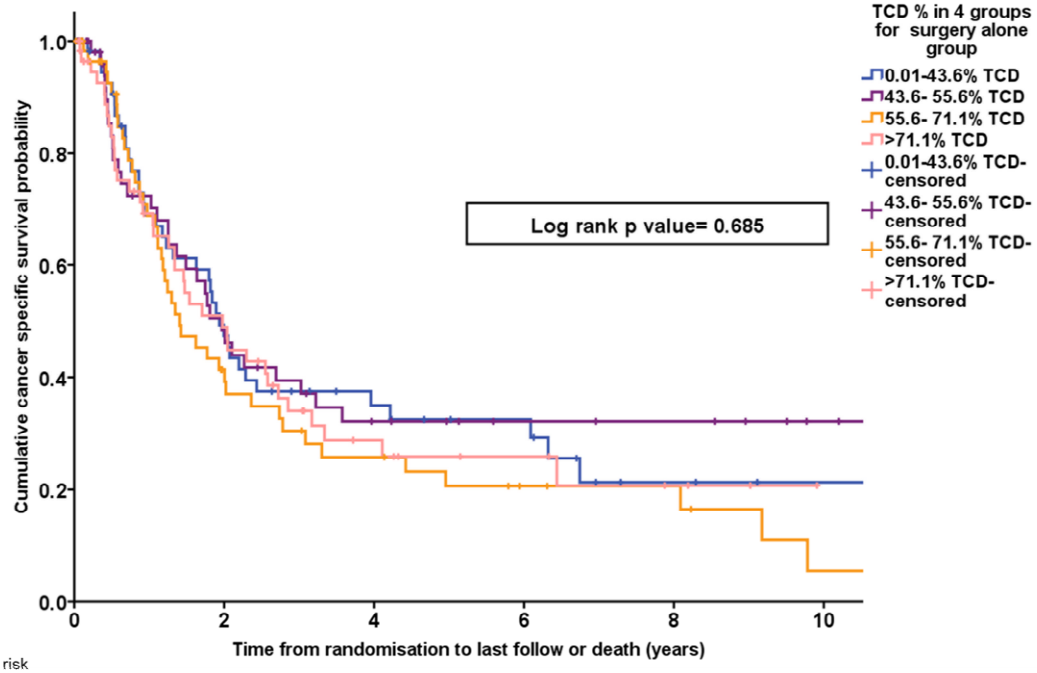
The four plots (a-d) assessing the prognostic value of TCD on overall survival by splitting the surgery alone treatment arm into 5 (a), 4 (b), 3 (c) or 2 (d) equally sized TCD groups. As the complete responders group only included 3 patients the curve has not been shown although patients at risk shown on life table below.

a.)



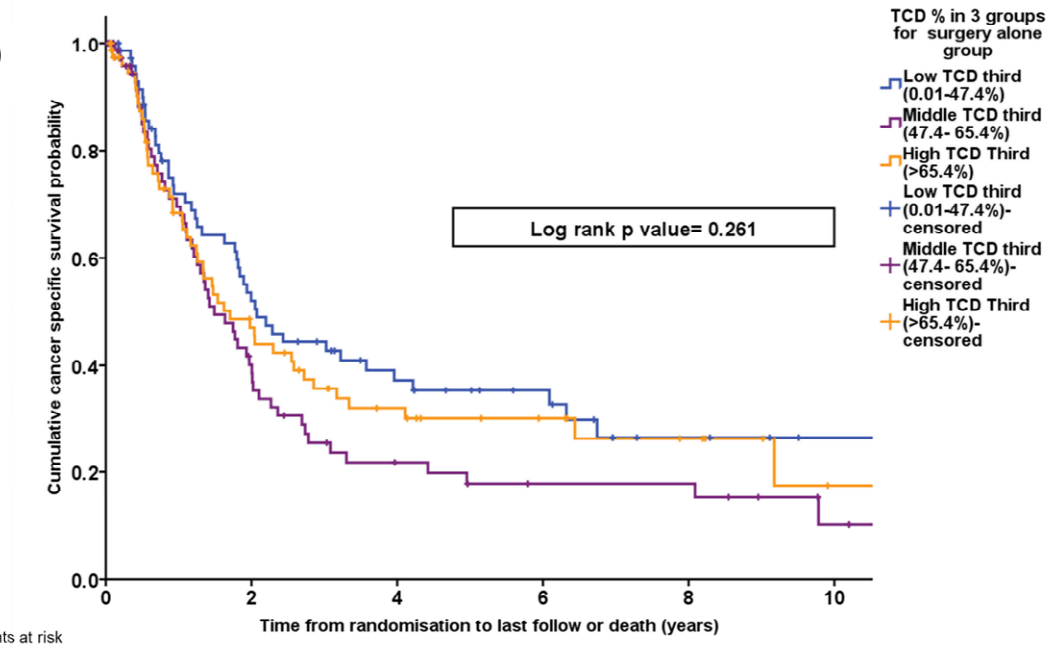
No. patients at risk						
Complete responders	3	1	1	1	1	1
0-39.6% TCD	46	19	11	8	3	1
39.6- 51.6% TCD	46	20	13	8	5	2
51.6-61.7% TCD	47	16	5	3	3	1
61.7- 74.8% TCD	48	13	10	6	4	1
>74.8% TCD	47	21	8	5	3	0

b.)



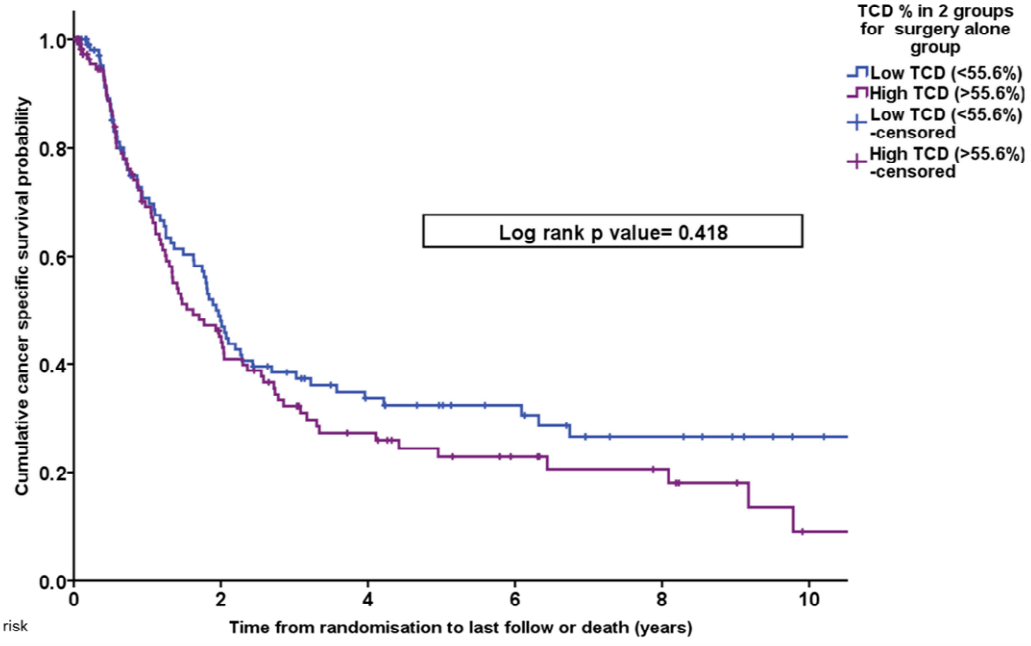
No. patients at risk						
Complete responders	3	1	1	1	1	1
1st quartile group	58	24	14	10	3	1
2nd quartile group	58	22	12	8	7	3
3rd quartile group	59	19	11	6	5	1
4th quartile group	59	24	10	6	3	0

c.)



No. patients at risk						
Complete responders	3	1	1	1	1	1
Low TCD third (<47.4%)	77	34	20	13	5	2
Mid TCD third (47.4-65.4%)	78	25	11	7	7	2
High TCD third (>65.4%)	79	30	16	10	6	1

d.)



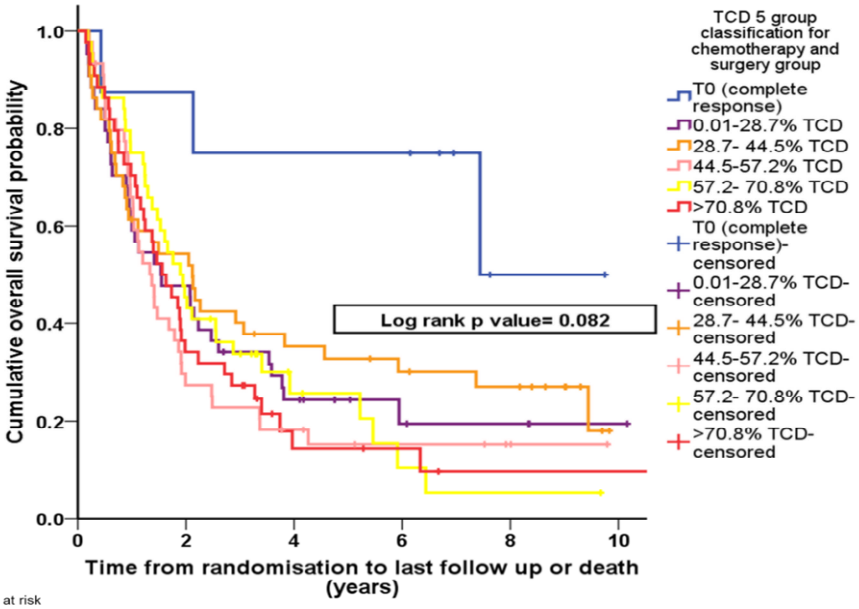
No. patients at risk						
Complete responder	3	1	1	1	1	1
Low TCD (<55.6%) group	116	46	26	18	10	4
High TCD (>55.6%) group	118	43	21	12	8	1

Figure 22 (a-d): Univariate cancer specific survival analysis by TCD grouping in the surgery alone treatment group.

The four plots (a-d) assessing the prognostic value of TCD on cancer specific survival by splitting the surgery alone treatment arm into 5 (a), 4 (b), 3 (c) or 2 (d) equally sized TCD groups. As the complete responders group only included 3 patients the curve has not been shown although patients at risk shown on life table below.

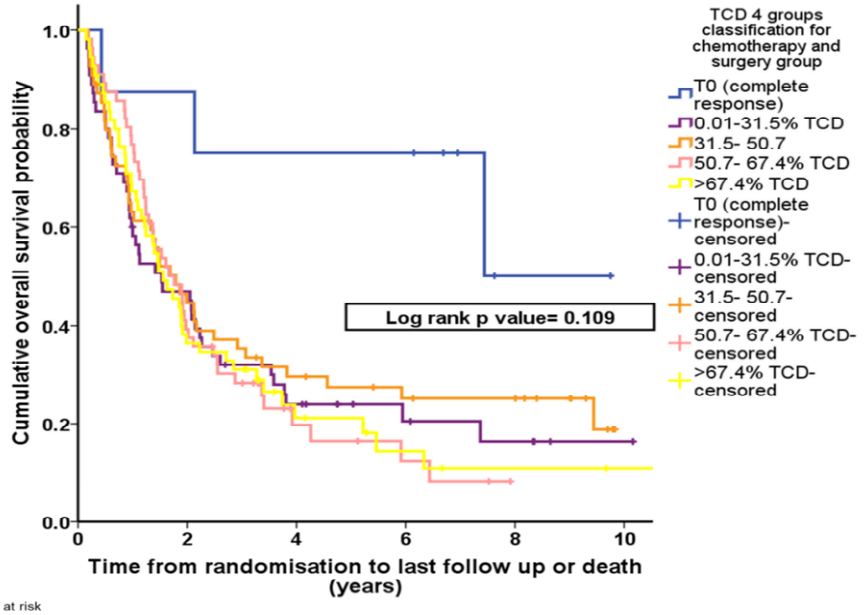
For the chemotherapy and surgery treatment group the TCD groupings do not produce significant differences in overall survival using the 5, 4 or 3 group system. However the 2 group system (cut off of 50.7%) produced a statistically significant differences in overall survival (p=0.031)(Figure 23a-d).

a.)



No. patients at risk						
Complete responder (T0)	8	7	6	6	1	0
0-28.7% TCD	44	21	10	4	3	1
28.7- 44.5% TCD	44	23	14	11	9	0
44.5-57.2% TCD	45	12	7	4	2	0
57.2- 70.8% TCD	44	20	6	2	1	0
>70.8% TCD	44	15	4	3	1	1

b.)



No. patients at risk						
Complete responder (T0)	8	7	6	6	1	0
1st quartile group	55	25	12	6	4	1
2nd quartile group	55	24	15	11	10	0
3rd quartile group	56	22	6	3	0	0
4th quartile group	55	20	8	4	2	1

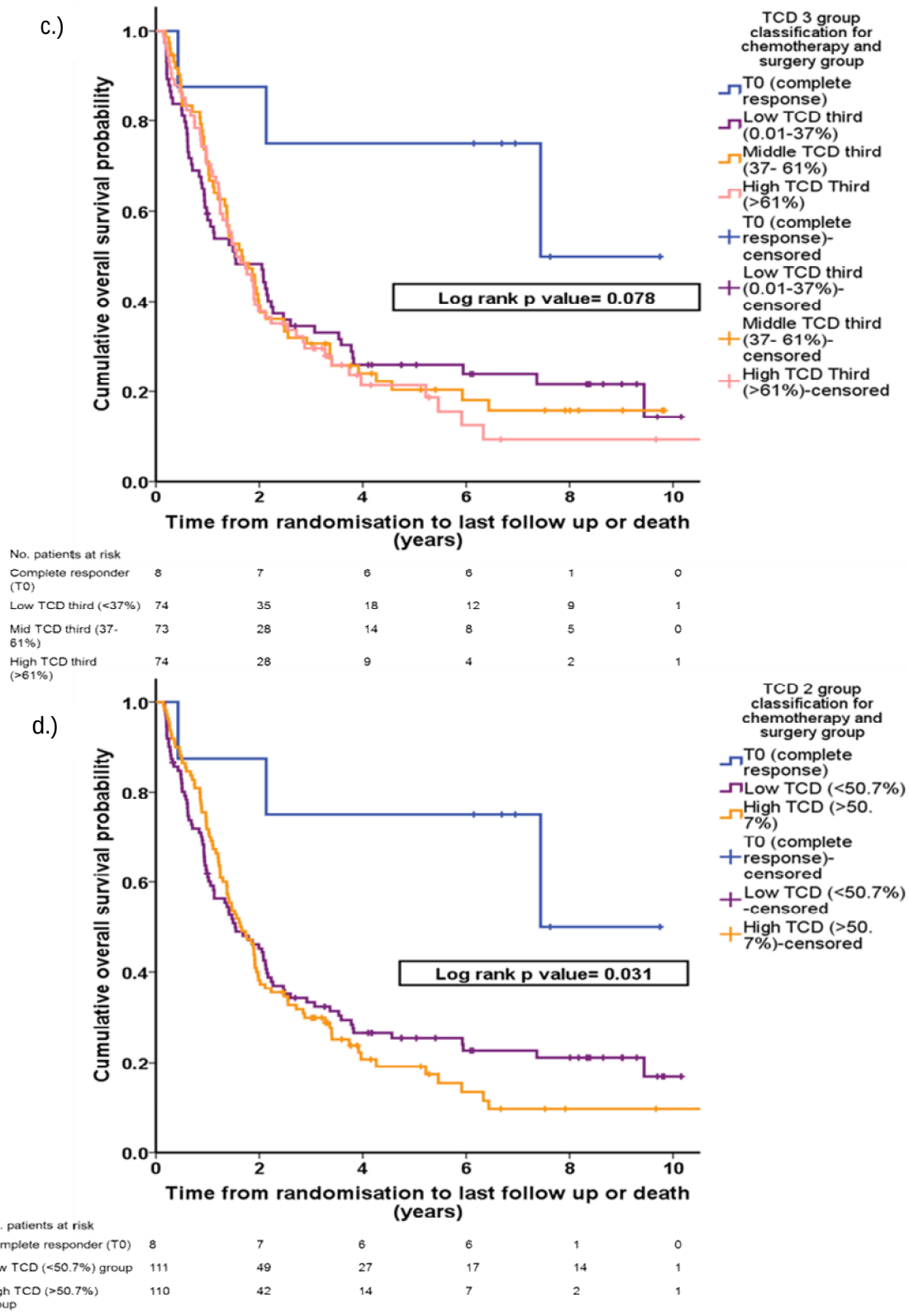
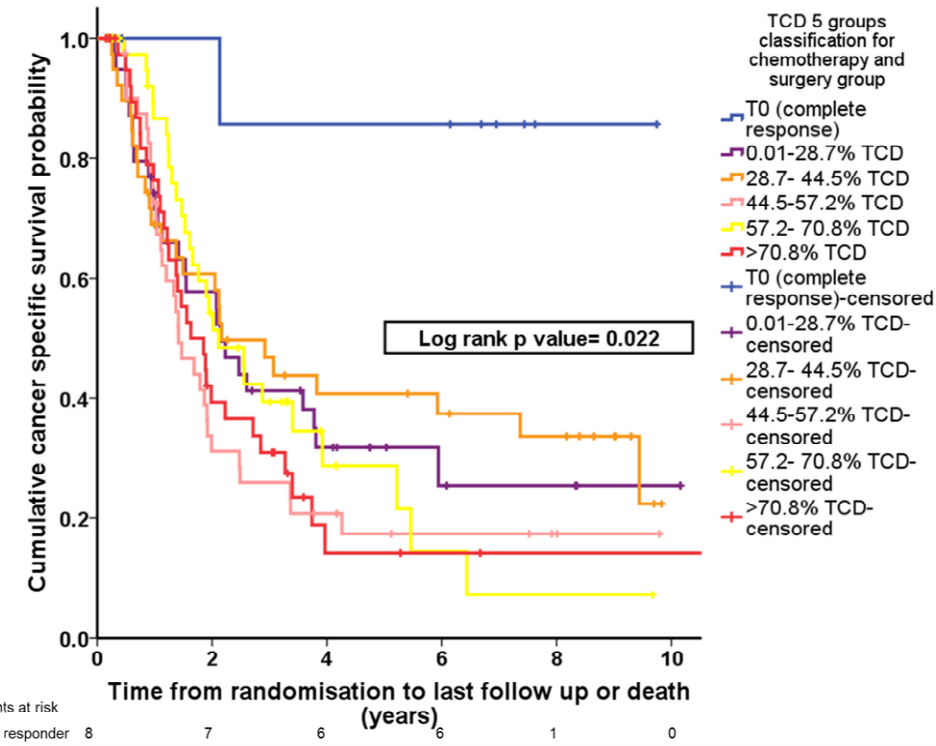


Figure 23 (a-d): **Univariate overall survival analysis by TCD grouping in the chemotherapy and surgery treatment group.**

The four plots (a-d) assessing the prognostic value of TCD on overall survival by splitting the chemotherapy and surgery treatment arm into 5 (a), 4 (b), 3 (c) or 2 (d) equally sized TCD groups. ypT0 cases are considered separately (n=8). The 2 group classification produced significant differences in survival (p=0.031).

However in cancer specific survival in the chemotherapy and surgery treatment arm 3 out of 4 of the grouping systems produced significant differences in survival between the groups and also the complete responders (Figure 24a-d). Unlike for overall survival log rank p values calculations showed these differences to be statistically significant in the 5 group classifications system (p=0.030) and 3 group system (p=0.032). Using a 2 group system (high >50.7% vs low <50.7%) showed the clearest differences in group survival particularly at the 8 year follow up time period (overall p=0.009). When comparing for differences only in the cases with evidence of residual tumour (i.e. excluding the complete responders n=8) there is no prognostic significance between the TCD groupings (5 groups p=0.320; 4 groups p=0.640; 3 groups p=0.521 and 2 groups p=0.264). This was also the case for overall survival when the complete responders were excluded none of the TCD group classifications was able to stratify patients.

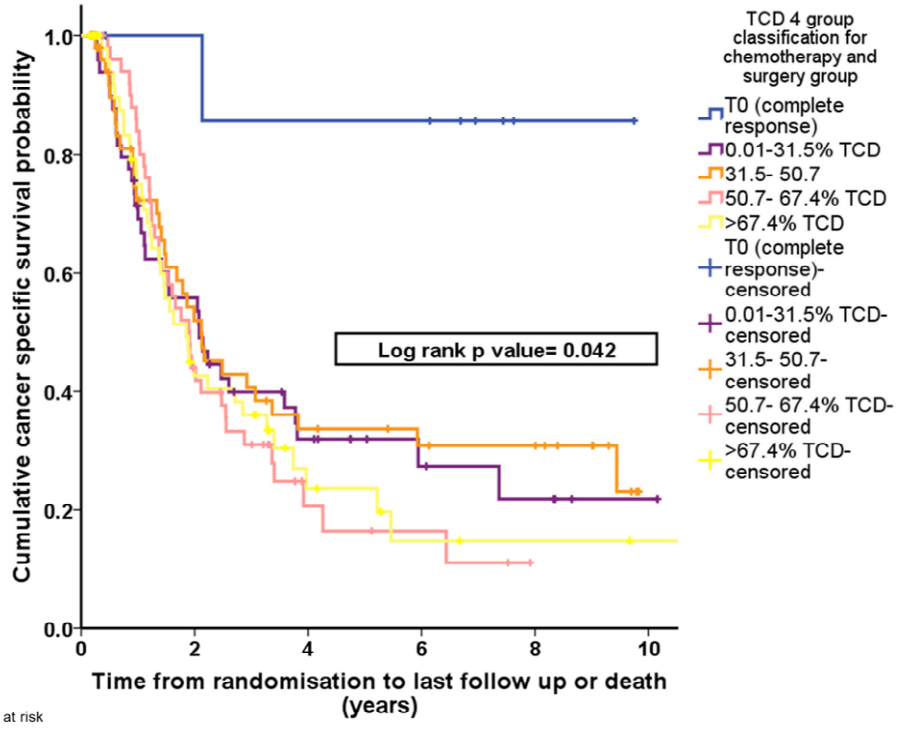
a.)



No. patients at risk

Complete responder (T0)	8	7	6	6	1	0
1st quartile group	55	25	12	6	4	1
2nd quartile group	54	23	14	11	10	0
3rd quartile group	55	21	5	3	0	0
4th quartile group	53	19	7	3	2	1

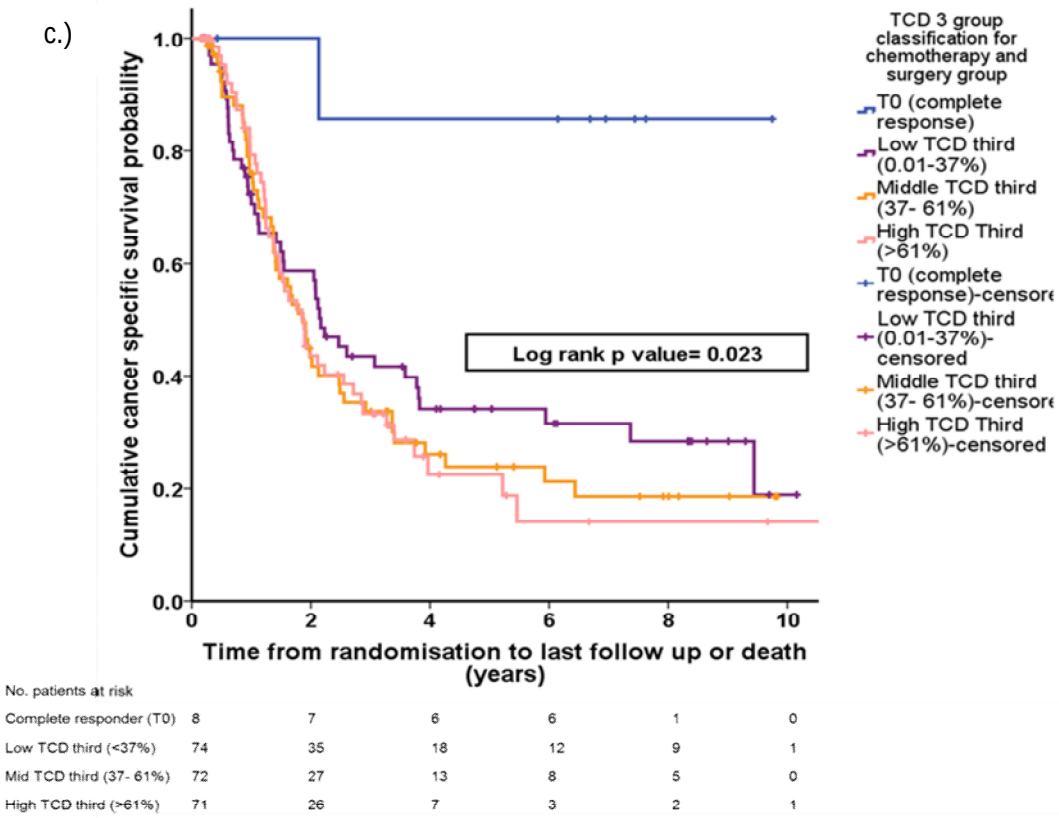
b.)



No. patients at risk

Complete responder (T0)	8	7	6	6	1	0
0-28.7% TCD	44	21	10	4	3	1
28.7- 44.5% TCD	43	22	13	11	9	0
44.5-57.2% TCD	45	12	7	4	2	0
57.2- 70.8% TCD	43	19	5	2	1	0
>70.8% TCD	42	14	3	2	1	1

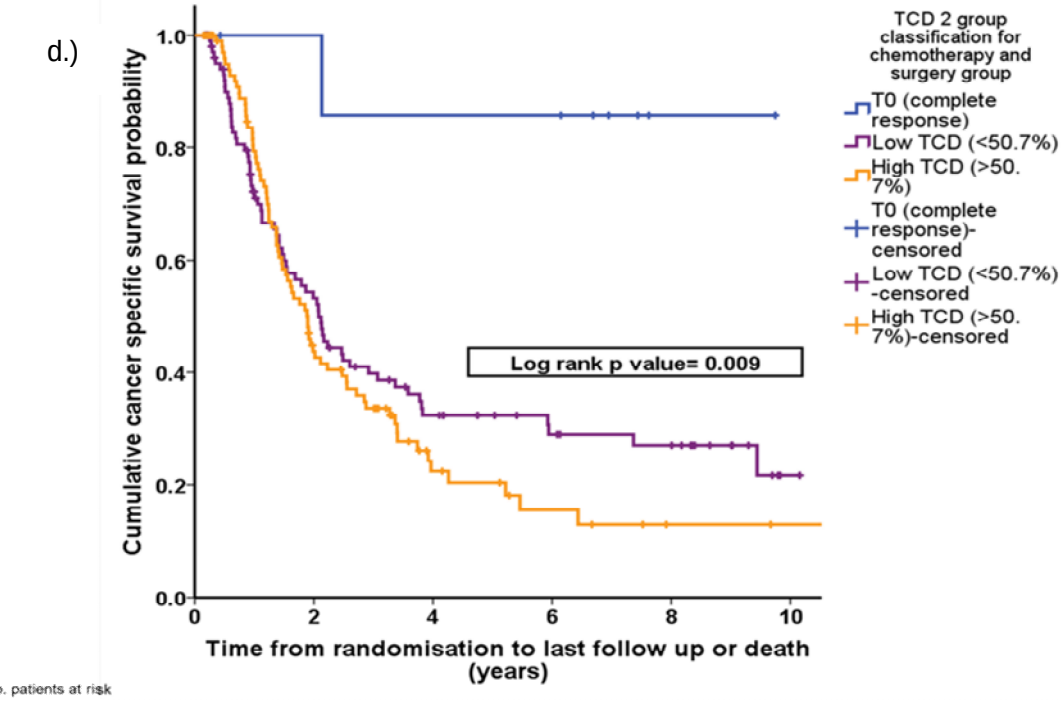
c.)



No. patients at risk

Complete responder (T0)	8	7	6	6	1	0
Low TCD third (<37%)	74	35	18	12	9	1
Mid TCD third (37- 61%)	72	27	13	8	5	0
High TCD third (>61%)	71	26	7	3	2	1

d.)



No. patients at risk

Complete responder (T0)	8	7	6	6	1	0
Low TCD (<50.7%) group	110	48	26	17	14	1
High TCD (>50.7%) group	107	40	12	6	2	1

Figure 24 (a-d): Univariate cancer specific survival analysis by TCD grouping in the chemotherapy and surgery treatment group.

The four plots (a-d) assessing the prognostic value of TCD on cancer specific survival by splitting the chemotherapy and surgery treatment arm into 5 (a), 4 (b), 3 (c) or 2 (d) equally sized TCD groups. Complete responders are considered separately (n=8) and their survival plot is also shown.

In the 3 group classification in chemotherapy and surgery treatment arm the medium (>37.1-61%) and high TCD group (>61%) survival probabilities are closely related throughout. So these two groups were combined to define a low group (TCD up to 37.1%) and a high group (TCD>37.1%). This modified 2 group classification also produced significant differences in survival for overall and cancer specific survival (p values 0.036 and 0.012 respectively) (Figure 25). However when just comparing the low vs high group of patients (excluding ypT0 patients) there was no statistical difference in survival between just the low and high group (overall survival p=0.678 and cancer specific survival p=0.257).

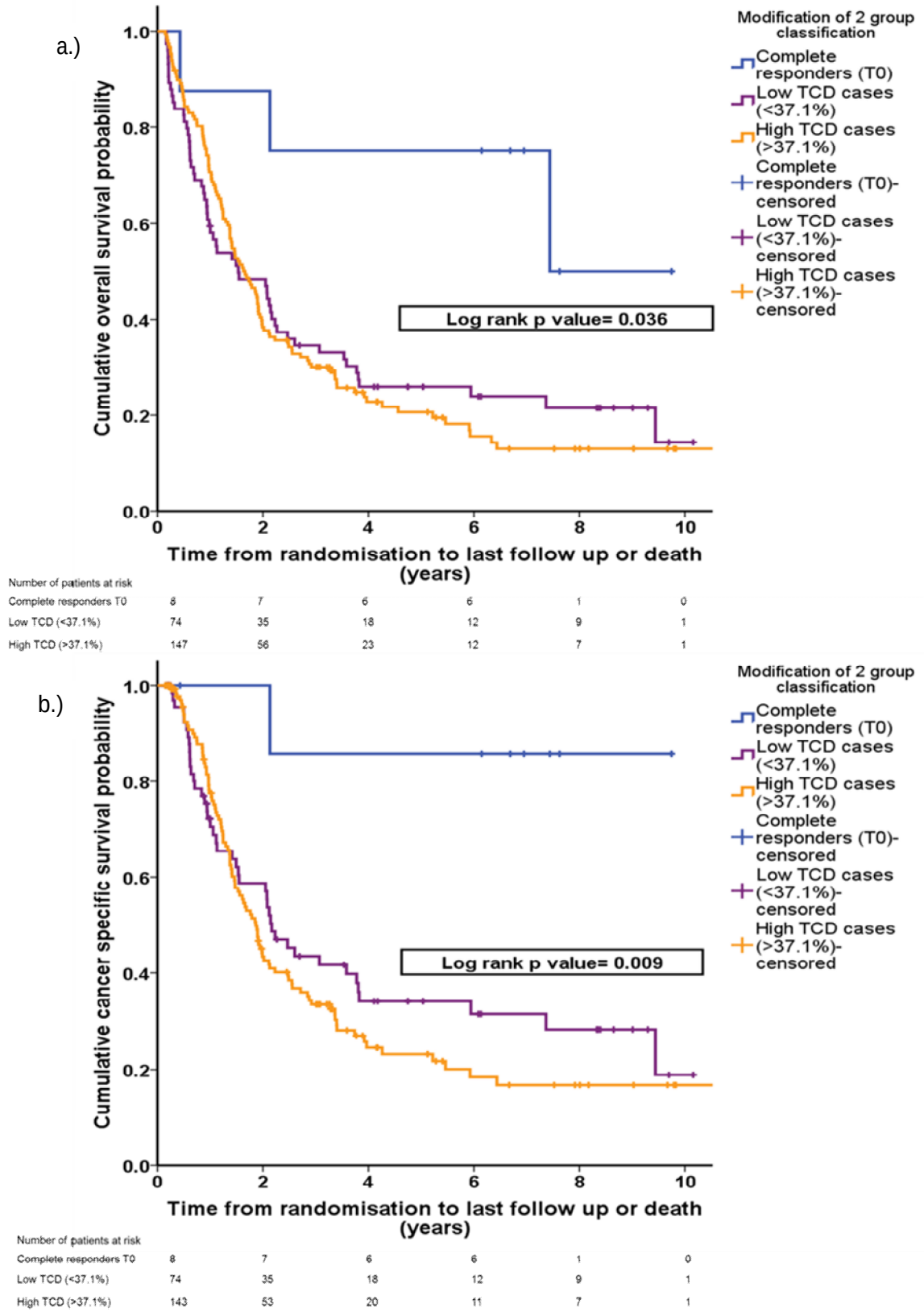


Figure 25 (a&b): Univariate overall and cancer specific survival analysis using the modified 2 group TCD classification for patients in the chemotherapy and surgery treatment group.

The survival plots of the patients from the chemotherapy and surgery treatment group using the modified 2 group classification. 24a shows the overall survival and 24b cancer specific survival.

Using this modified classification system (≤37.1% or >37.1% TCD), the patients from the chemotherapy and surgery treatment group (n=229) clinicopathological demographics were compared, results can be found in Table 20. Patients with low TCD tumours (0-37.1% TCD) were significantly more frequently classified as Mandard TRG 2 or 3 compared to patients with high TCD tumours (>37.1% TCD). 51.8% of patients with high TCD tumours had a Mandard TRG 5 (no evidence of regression) compared to 34.2% of patients with low TCD tumours.

Table 20: TCD high and low groups clinicopathological distributions.
The distribution of the clinicopathological features from the chemotherapy followed by surgery patients using the low and high modified TCD groups (Figure 25).

	Complete responders (n=8)	Low TCD (≤37.1%) (n=74)	High TCD (> 37.1%) (n=147)	
	Number (%)	Number (%)	Number (%)	P[1] value
Age				
Median	62.4	63.2	60.8	0.034
Range	42.4- 75.5	36.4- 83.1	36.8- 77.7	
Gender				
Male	4 (60.0)	56 (75.7)	122 (83.0)	0.196
Female	4 (40.0)	18 (24.3)	25 (17.0)	
Histology				
Adenocarcinoma	0 (0.0)	49 (66.2)	114 (77.6)	0.073
Squamous	0 (0.0)	22 (29.7)	29 (19.7)	
Other	0 (0.0)	3 (4.1)	4 (2.7)	
No residual tumour	8 (100.0)	0 (0.0)	0 (0.0)	
Differentiation				
Poor	0 (0.0)	36 (48.6)	56 (38.1)	0.098
Moderate	0 (0.0)	34 (45.9)	75 (51.0)	
Well	0 (0.0)	4 (5.4)	14 (9.5)	
Unknown	0 (0.0)	0 (0.0)	1 (0.7)	
No residual	8 (100.0)	0 (0.0)	0 (0.0)	
T category				
T0 (No residual)	8 (100.0)	0 (0.0)	0 (0.0)	0.856
T1	0 (0.0)	6 (8.1)	13 (8.8)	
T2	0 (0.0)	11 (14.9)	18 (12.2)	
T3	0 (0.0)	54 (73.0)	115 (78.2)	
T4	0 (0.0)	3 (4.1)	1 (0.7)	
N category				
N0	8 (100.0)	35 (47.3)	58 (39.5)	0.266
N1	0 (0.0)	39 (52.7)	89 (60.5)	
Lymphatic invasion				
Yes	0 (0.0)	15 (20.3)	46 (31.3)	0.115
No	8 (100.0)	53 (71.6)	88 (59.9)	
Suspicious	0 (0.0)	6 (8.1)	13 (8.8)	
Vascular invasion				
Yes	0 (0.0)	5 (6.8)	11 (7.5)	0.657
No	8 (100.0)	63 (85.1)	128 (87.1)	
Suspicious	0 (0.0)	6 (8.1)	8 (5.4)	
Mandard grading*				
TRG1	8 (100.0)	0 (0.0)	0 (0.0)	***<0.001***
TRG2	0 (0.0)	5 (6.8)	2 (1.4)	
TRG3	0 (0.0)	16 (21.6)	10 (6.8)	
TRG4	0 (0.0)	36 (48.6)	57 (38.8)	
TRG5	0 (0.0)	17 (23.0)	78 (53.1)	

p[1] values- Calculated using Mann-Whitney test
* Mandard grading; TRG 1= complete regression, TRG2= only a few residual tumour cells evident, TRG3= predominantly fibrosis, TRG4= predominantly tumour cells and TRG5= no evidence of any regression seen.

4.7.3 Assessment of the prognostic value of TCD in univariate and multivariate analysis

The results of cox regression testing for overall survival in the surgery alone group is outlined in Table 21 below, identifying patient age, T category, N category and presence of lymphatic invasion as prognostic factors. TCD was not related to overall survival in the surgery alone patients (p=0.960). On multivariate analysis, patients age (p=0.005), N category (p<0.001) and evidence of lymphatic vessel invasion (p=0.012) were identified as independent prognostic markers for overall survival (Table 21).

Table 21: Surgery alone patients overall survival cox regression analysis table.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P value	HR	95% CI	*P value
Age	1.02	1.01 to 1.04	***0.005***	1.02	1.01 to 1.04	***0.002***
Gender	0.84	0.61 to 1.15	0.274	-	-	-
Histology	1.03	0.84 to 1.26	0.772	-	-	-
Differentiation	1.00	1.00 to 1.00	0.572	-	-	-
T category	1.43	1.15 to 1.76	***0.001***	1.20	0.94 to 1.54	0.151
N category	2.15	1.57 to 2.94	***<0.001***	1.85	1.33 to 2.59	***<0.001***
Lymphatic involvement	1.39	1.13 to 1.70	***0.002***	1.33	1.07 to 1.66	***0.012***
Blood vessel involvement	1.20	0.98 to 1.47	0.083	-	-	-
TCD%	1.00	0.85 to 1.18	0.960	-	-	-

The relationship between age, gender, histology, grade of tumour differentiation, pT, pN categories, lymphatic involvement, blood vessel involvement and TCD to cancer specific survival was also assessed via cox regression analysis. The ypT category, ypN category, lymphatic vessel invasion and blood vessel invasion were significant

prognostic marker in univariate analysis (Table 22). Multivariate analysis showed pT category, pN category and lymphatic vessel invasion as independent prognostic markers (Table 22).

Table 22: Surgery alone patients cancer specific survival cox regression analysis table.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	**P* value
Age	1.01	1.00 to 1.03	0.115	-	-	-
Gender	0.75	0.52 to 1.08	0.124	-	-	-
Histology	1.12	0.80 to 1.57	0.505	-	-	-
Differentiation	1.00	1.00 to 1.00	0.705	-	-	-
T category	1.81	1.36 to 2.41	***<0.001***	1.41	1.01 to 1.97	***0.041***
N category	2.91	1.99 to 4.28	***<0.001***	2.21	1.47 to 3.34	***<0.001***
Lymphatic involvement	1.60	1.27 to 2.01	***<0.001***	1.50	1.16 to 1.93	***0.002***
Blood vessel involvement	1.31	1.05 to 1.63	***0.017***	1.11	0.88 to 1.41	0.363
Overall TCD%	1.00	1.00 to 1.01	0.370	-	-	-

The same cox regression analysis were performed on the survival data for patients from the chemotherapy and surgery group (n=229). As this treatment group involved neoadjuvant chemotherapy patients, the Mandard TRG system was also included in the model to assess its prognostic value.

Univariate overall survival analysis identified age, ypT category, ypN category, lymphatic vessel invasion, blood vessel involvement, TCD and Mandard TRG as prognostic markers (Table 23). On multivariate analysis age, ypT category, ypN

category and blood vessel involvement were found to be independent prognostic marker (Table 23).

Table 23: Chemotherapy and surgery treatment group overall survival Cox regression.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	**P* value
Age	1.02	1.00 to 1.04	***0.016***	1.02	1.00 to 1.04	***0.013***
Gender	0.973	0.68 to 1.40	0.88	-	-	-
Histology	0.91	0.77 to 1.09	0.313	-	-	-
Differentiation	1.00	1.00 to 1.00	0.642	-	-	-
T category	1.59	1.30 to 1.95	***<0.001***	1.41	1.13 to 1.76	***0.002***
N category	1.95	1.44 to 2.63	***<0.001***	1.63	1.18 to 2.24	***0.003***
Lymphatic involvement	1.30	1.05 to 1.60	***0.015***	1.15	0.91 to 1.45	0.240
Blood vessel involvement	1.54	1.21 to 1.97	***0.001***	1.42	1.10 to 1.84	***0.008***
Mandard Grade	1.22	1.05 to 1.40	***0.008***	0.96	0.79 to 1.16	0.675
Overall TCD%	1.01	1.00 to 1.01	***0.047***	1.00	1.00 to 1.01	0.530

The relationship between age, gender, histology, grade of tumour differentiation, ypT, ypN categories, lymphatic involvement, blood vessel involvement and TCD to cancer specific survival in the chemotherapy and surgery treatment group, was also assessed via cox regression analysis (Table 24).

Table 24: Cancer specific Cox regression analysis in chemotherapy and surgery group

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	**P* value
Age	1.02	1.00 to 1.04	***0.038***	1.02	1.00 to 1.04	***0.024***
Gender	0.97	0.65 to 1.46	0.898	-	-	-
Histology	0.83	0.68 to 1.03	0.086	-	-	-
Differentiation	1.05	0.93 to 1.17	0.437	-	-	-
T category	1.95	1.50 to 2.53	***<0.001***	1.61	1.22 to 2.14	***0.001***
N category	2.44	1.72 to 3.47	***<0.001***	1.96	1.35 to 2.83	***<0.001***
Lymphatic involvement	1.30	1.03 to 1.65	***0.028***	1.11	0.85 to 1.45	0.433
Blood vessel involvement	1.68	1.29 to 2.19	***<0.001***	1.53	1.15 to 2.03	***0.003***
Mandard Grade	1.34	1.13 to 1.59	***0.001***	1.00	0.80 to 1.26	0.990
TCD%	1.01	1.00 to 1.02	***0.009***	1.00	1.00 to 1.01	0.443

There was a significant relationship between cancer specific survival and age, ypT category, ypN category, lymphatic vessel invasion, blood vessel involvement, Mandard TRG and TCD % (Table 24). Multivariate analysis identified age, ypT category, ypN category and evidence of blood vessel involvement as independent prognostic markers for cancer specific survival.

4.8 Comparison of the prognostic value of tumour cell density scoring versus Mandard tumour regression grading in chemotherapy followed by surgery patients

To further assess the value of the TCD measurement as a surrogate of tumour regression, TCD results were compared to the results from the Mandard tumour regression grading for patients from the chemotherapy and surgery treatment group (Figure 26). TCD % values were taken as a continuous variable with the ypT0 cases included (n=8) with their score given as 0% TCD. Kruskal Wallis test showed that a significant relationship between TCD % and Mandard grading ($p<0.001$)

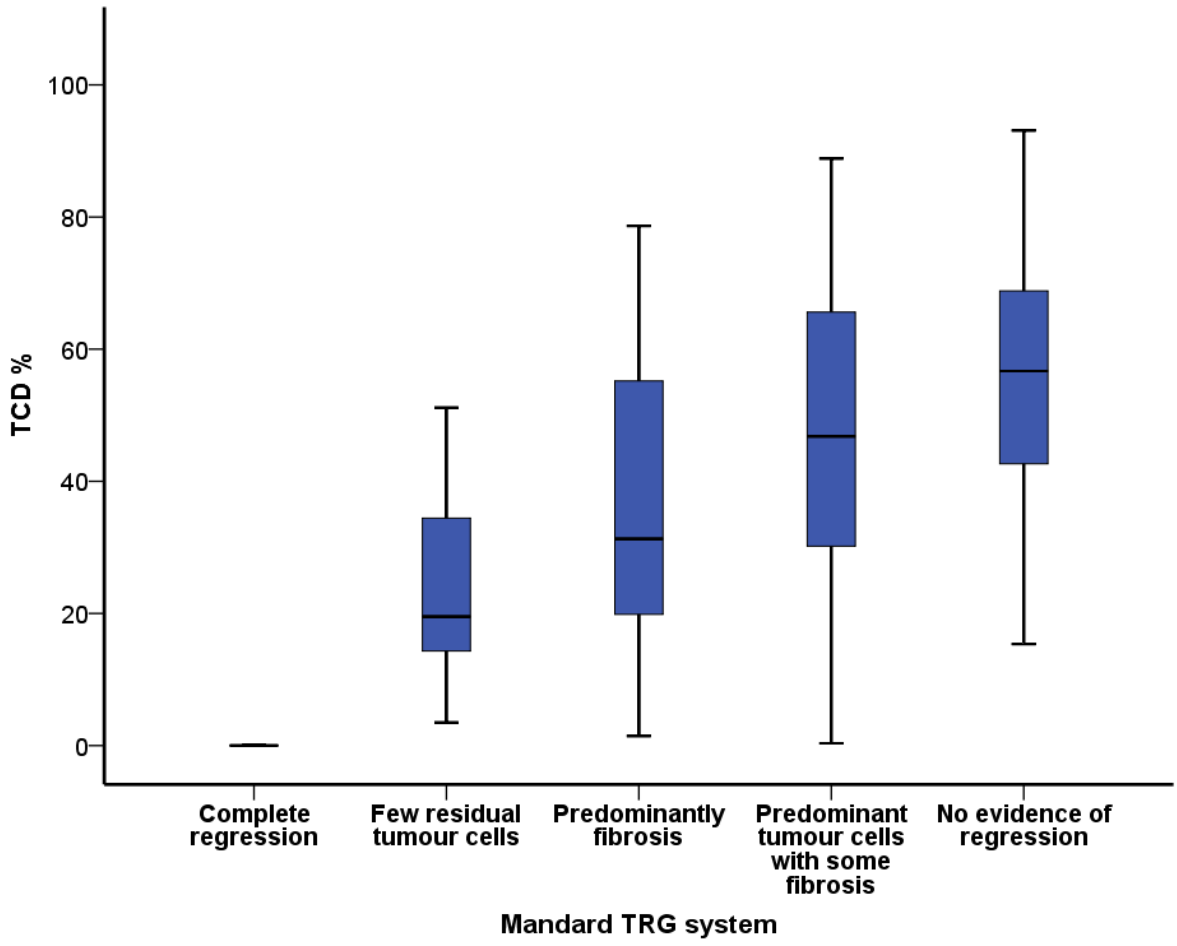
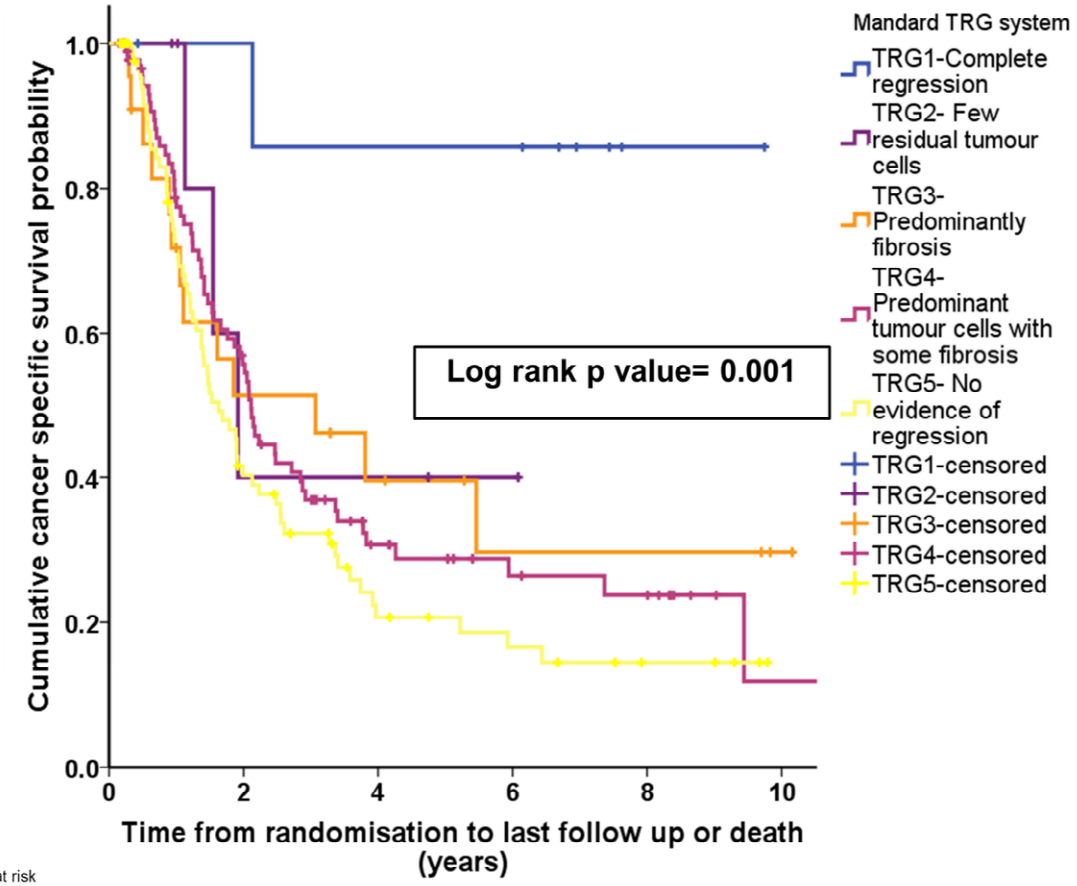


Figure 26: Boxplot showing a linear relationship between TCD % and Mandard tumour regression grading system

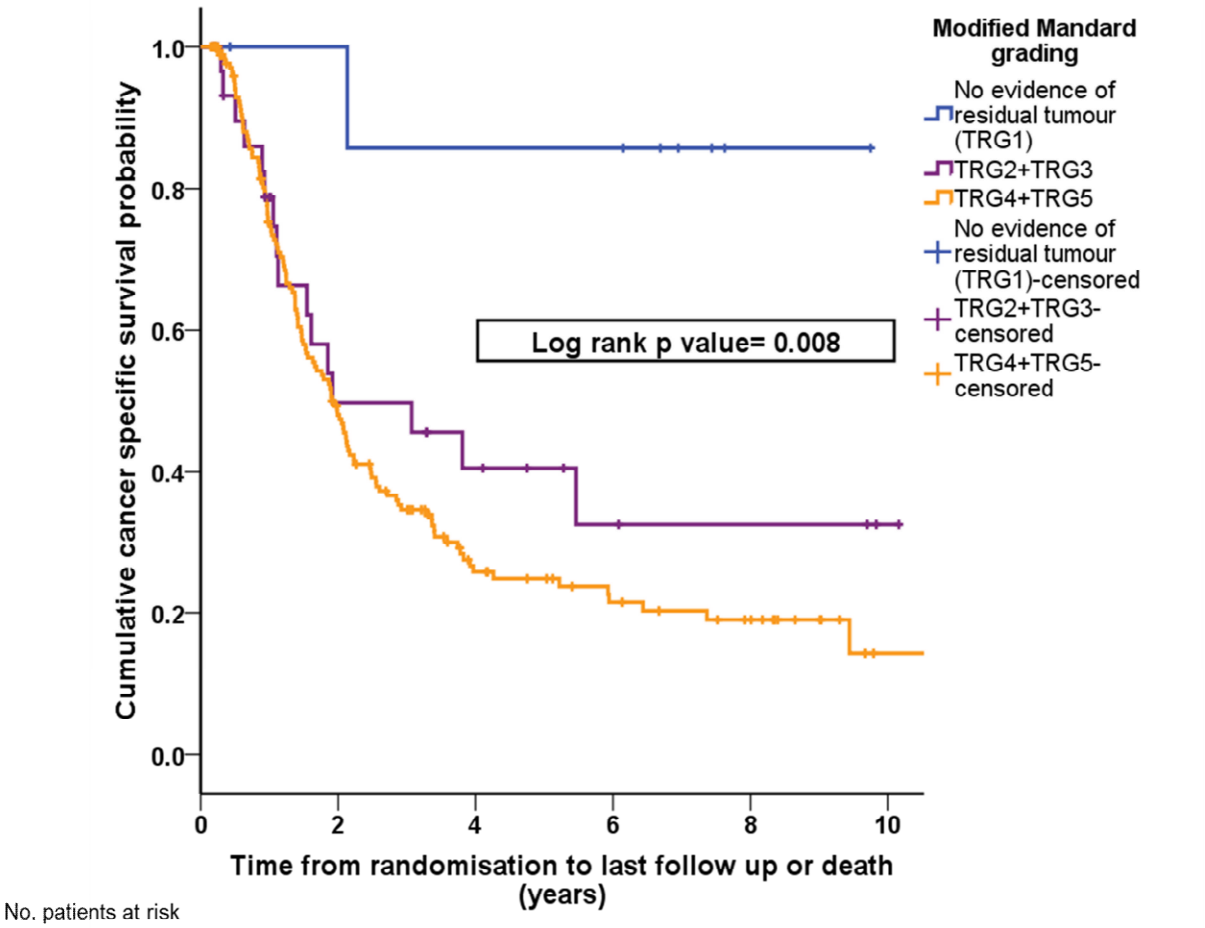
Univariate analysis showed TCD % to be prognostic in chemotherapy and surgery patients for overall (HR 1.01; CI 1.00 to 1.01; p=0.047) and cancer specific survival (HR 1.01; CI 1.00 to 1.02; p=0.009). Mandard TRG was also found to be of prognostic value in univariate analysis for chemotherapy followed by surgery patients for overall (HR 1.22; CI 1.05 to 1.40; p=0.008) and cancer specific survival (HR 1.34; CI 1.13 to 1.59; p=0.001) (Figure 27). However neither TCD% nor Mandard TRG was demonstrated to be independent prognostic markers.



No. patients at risk						
Complete regression (TRG1)	8	7	6	6	1	0
Few residual tumour cells (TRG2)	7	2	2	1	0	0
Predominant fibrosis (TRG3)	26	10	6	3	3	1
Predominant tumour (TRG4)	92	45	18	11	9	1
No evidence of regression (TRG5)	92	31	12	8	4	0

Figure 27: Kaplan Meier survival plot of cancer specific survival in the chemotherapy and surgery treatment group stratified by Mandard tumour regression grading.

The Kaplan Meier plot was visually inspected to identify whether some of the groups should be combined as they seem to have the same survival. Therefore TRG 1 was compared with TRG2+3 combined and TRG 4+5 combined (Figure 28).



No. patients at risk

TRG1	8	7	6	6	1	0
TRG2+3	33	12	8	4	3	1
TRG4+5	184	76	30	19	13	1

Figure 28: Cancer specific survival in the chemotherapy and surgery treatment group stratified by a modified Mandard regression grading system.

Using this modified Mandard tumour regression grading system, a significant difference in cancer specific survival was seen (p=0.008). However as with TCD when the univariate survival is assessed without the complete responders (i.e. TRG1 cases not included) there is no significant difference in survival between the patients with TRG2 or 3 compared to patients with TRG 4 or 5 (p=0.231).

4.9 Discussion

The OE02 trial and subsequently published meta-analyses showed that neoadjuvant chemo(radio)therapy improved prognosis in oesophageal cancer patients prognosis (94, 99, 335, 336). Tumour regression has been shown to have prognostic value in oesophageal cancer patients who have undergone (radio)chemotherapy (121, 129, 174, 178, 335, 337-340), with cases showing complete regression having the greatest survival benefit (121, 129). Some studies have stated histological response was an independent prognostic marker for survival (175, 176, 341, 342). At present, response to neoadjuvant chemotherapy during treatment is measured radiologically on CT imaging using the RECIST criteria, with variable degree of confidence. However, except for RECIST, which is a radiological regression system, there is no universally accepted histopathological response evaluation system established (175, 341).

Pathological review of the resected tumour following neoadjuvant therapy remains the current gold standard for the assessment of response to therapy (174, 186). Current tumour regression grading systems use subjective measures to determine either the amount of regression (177) or the percentage of residual tumour (175, 176, 179, 189) or the size of the residual tumour foci (190). In the UK, the tumour regression grading (TRG) system described by Mandard et al is widely used which was originally developed to assess response in squamous cell cancers after chemoradiotherapy (177) . Mandard TRG has been criticised as being a subjective method with particular high inter-observer variation for grading of tumours with reduced number of tumour cells (175, 176, 340). There is also controversy on how many different categories should be used with Mandard and Dunne favouring the 5 group classification (177, 189), while others demonstrating a better prognostic stratification using a 4 group (176, 186, 194), and some investigators more recently suggesting a 3 grade system being superior (121, 186, 335). Swisher et al proposed a 3 grade tumour regression grading system consisting of P0 (complete responders), P1 (1-

50% residual tumour) and P2 (greater than 50% residual tumour) and indicated that the use of a pathological category should be added into the TNM classification (186).

The work in this study has used quantitative measurements of the tumour cell density to provide an assessment of the tumour regression. The tumour cell density (TCD) has been calculated at the luminal surface of oesophageal cancer resection specimens using virtual H&E stained sections to simulate endoscopic biopsies and been shown to have prognostic value in the chemotherapy followed by surgery treatment group. The TCD measurement technique was based on a well-established morphometric method called point counting which provides in this case a quantitative measure for tumour cell density of tumours (330). Through the intra-observer variability analysis the technique has been shown to be reproducible with a new observer learning curve demonstrated with an improvement in intra-observer scoring noted through the process. Inter-observer analysis has shown a good agreement between two independent observers (TS and GH) with a Kappa calculated agreement of 0.732, demonstrating the scoring system used as shown previously can be followed closely by different observers (198). Kappa coefficient gives a measure of agreement and a kappa agreement above 0.70 is generally accepted as good. This is especially considering this agreement was between a qualified histopathologist and a second observer with no formal histopathological qualifications, who was pre-trained in the technique and developed further through the scoring process.

This TCD technique has been shown to be significantly related to Mandard grading through non-parametric testing ($p<0.001$), with box plot comparisons show that TCD values correlate well with Mandard grades. Mandard grading is widely used in the UK for reporting of regression in oesophageal resections. Survival analysis shows both systems to stratify patients into prognostic groups. However using TCD calculations give the advantage of producing quantitative measurements therefore reduce subjective bias. As well as this TCD

calculations can be done on biopsies at the pre-operative category and through serial biopsies could be used to assess response through neoadjuvant chemotherapy.

The TCD % demonstrated a range from 0-95.5% with a normal distribution across the cohort as a whole. There was a statistically significant difference in the TCD scoring between the treatment arms showing that the regression changes induced by chemotherapy did change the actual tumour density and that this change can be quantified at the luminal surface. Some studies have shown that amount of residual tumour cells in OeC is significantly associated with prognosis but no study has gone on to use quantitative scoring of the residual tumour density as done in this study (180, 325). However here tumour cell density is measured as a continuous variable in larger group of patients (n=469), originating from a large multi-centre randomised trial (99).Only one previous study in OeC has looked at the tumour cell density in surgery alone patients. This study by Mesker et al showed that even without neoadjuvant therapy OeC can have varying densities in their resection with 35% of cases having less than 50% tumour to stroma. This work reflects this finding as not all cases in the surgery alone group had high tumour densities with the median TCD in this group being 55.6% (range 0.3 to 95%). This has been described before in surgery alone tumours with abundant stromal tissue leading to lower tumour cell densities (191, 325). In this study 97 (39%) cases in the surgery alone group had a TCD<51% which using established regression grading systems such as Mandard's or Becker's would classify these patients as "responders" despite never having any neoadjuvant treatment (176, 177). This may suggest that in the neoadjuvant chemotherapy followed by surgery group, the resection tumour density may have not necessarily decreased or changed at all as a result of chemotherapy. This supports assessing the TCD early in the treatment pathway to establish the tumours original TCD. Responders will then be cases where the TCD shows a significant decrease from its original (pre-chemotherapy) TCD to its final resection TCD%.

TCD is shown in this chapter to be a prognostic survival marker, only in chemotherapy followed by surgery patients and not in surgery alone patients. TCD has been identified as independent prognostic marker in previous studies in breast (343), colorectal (198, 344) and also oesophageal cancer (325). This previous tumour regression study involving oesophageal cancer patients was by Mesker et al examining the “tumour to stroma” ratio as opposed to tumour cell density used here (325). Mesker’s study (n=93) also differed from this current one because it only included patients’ who had undergone surgery alone, measured the overall tumour cells, therefore not at the luminal surface as in this study and also this initial study used estimations of the tumour amount as opposed to the quantitative measures carried out in this work (325). Cases were divided into those with low tumour stroma ratio (<50%) and those with high tumour stroma ratio (>50%). Patients in the high tumour ratio group had a significantly better median overall survival (42 months) compared to those in the low tumour stroma group (16 months) which was statistically significant ($p<0.001$). This would suggest that patients with less than 50% tumour cells had a poorer prognosis than those with high tumour cells, which is in opposition to our study. However the scoring done in Mesker et al was done in areas with the lowest tumour to stroma ratio which they describe as generally at the deepest point of infiltration (325). Mesker followed up this initial study with another assessing tumour to stroma in oesophageal cancer biopsy tissue using patients who had only undergone surgery (196). This demonstrates that tumour density measure could potentially be done on biopsy material but this time with a quantitative measurement as done in this study.

Our TRG grouping tests have identified a similar cut-off of 50.7% TCD to split the cohort into 2 equal groups (low vs high TCD) as used by other tumour regression systems (175, 178, 185, 186) (Table 19). Univariate survival analysis shows this cut-off to give groups with significant differences in cancer specific survival but only when the ypT0 cases are included separately. However using TCD enables a quantitative measurement to be carried out as

opposed to estimations used in regression grades such as Mandard, Becker or Chirieac et al (177-179). Another difference with using this TCD technique is that it enables a measurement to be carried out at the luminal surface a site that could be biopsied in the pre-operative phase of treatment, while all other regression techniques are limited to the assessment of resected cancers. Although not specifically tested for in this work the use of TCD calculations through serial biopsies could provide an important marker to monitor oesophageal cancer during chemotherapy and has been shown in previous oesophageal cancer studies (196).

While TCD was not shown to be an independent prognostic marker in chemotherapy and surgery, ypT and ypN categories were independently prognostic using TNM6 (140). This finding is in agreement with a large recent study looking at prognostic markers in oesophageal cancer (345) . The study of 850 oesophago-gastric cancers concluded that ypTNM stages were the only independent prognostic factors. The regression grading tested was the Becker 4 tier regression grading system which was shown to have prognostic value but not to be independently prognostic. However there remains other studies who have found tumour regression grading in oesophageal cancer to be an independent prognostic marker and have suggested that histopathological response should be incorporated into the TNM classification (185, 186, 196). These contrasting results are likely due to multiple factors between the studies. Firstly, there are a variety of different regression systems used across existing studies (discussed in 1.9 Tumour regression systems used to assess oesophageal cancer) with regional preferences found (177-179). Different systems have different cut-offs and involve different number of classification groups making study results difficult to compare (177-179, 185, 189). However all have the same limitation of requiring estimations of regression. Also there are differences in the histological type of the oesophageal cancer cases included in the analysis with some studies only involving squamous cell carcinomas (176, 177, 195), some involving only adenocarcinomas (175,

325, 342) and others have a mixed histological cohort (121, 187). Although it has previously been suggested that survival differs between the two main histological oesophageal cancer types (127, 129), in this study non-parametric testing showed no significant difference in TCD scoring between adenocarcinomas and squamous cell carcinomas. This study to date will be the first to include patients from a randomised control trial in oesophageal cancer with patients who have had both surgery alone and neoadjuvant chemotherapy followed by surgery which will be the last time such a cohort will be used following the results of the OE02 trial (84). This study is also different to all previous studies as it is the first to measure the TCD specifically at the luminal surface in an attempt to compare this TCD value to calculations that could be taken from endoscopic biopsies.

Despite having unique features this study did have limitations to acknowledge, firstly, although the scoring labels were clear and inter observer agreement good there were still times where the labelling of points could be due to subjective judgement. Except for cases reviewed as part of the inter-observer analysis all cases were only reviewed by one single observer (TS). Although a second observer to review each case would have been ideal the time consuming nature of reviewing 469 cases limited this option. Secondly, all added pathological variables were done using the material received for each case by a local histopathologist (NW). As with the 28 cases where no tumour involving material was received it is likely that even in cases with tumour present not all the cases material was available. This could have influenced the added information gained locally such as lymphatic vessel or blood vessel involvement as well as Mandard grading if it was based on incomplete material. Finally the criteria for selection of appropriate slide could also be criticised for using the slide with the deepest infiltration and not the slide with the greatest density as the first selection rule. This was aimed to be associated with where the tumour would possibly have the highest pT category, but with the possibility of not all the slides being available this may not have been the case. The reason the slide with the greatest

density of visible tumour was not used as the first criteria was because this could have added an element of subjectivity to the slide selection process. The good intra-observer agreement in terms of slide selection is reassuring. Ideally this work would have included some TCD values from the relevant biopsies of each case. However as not all this material was available this was not possible; however this would be a likely follow up study in future.

This study has shown that morphometric point counting technique is an easy, reproducible technique which enables quantitative measurements of the TCD in oesophageal cancer. TCD calculation has been shown to correlates to with the internationally recognised Mandard grade with both on univariate analysis demonstrating prognostic value in chemotherapy followed by surgery oesophageal cancer. However using TCD has the advantage of being an objective measurement through a quantitative calculation of residual tumour present. This TCD method has been shown to demonstrate a difference in tumour cell density after chemotherapy, a change which can be measured at the luminal surface. This finding suggests that TCD measurements could be used to assess tumour response during the pre-operative phase via endoscopic biopsies which would be the next logical study to be carried out. With particular interest would be if it’s the actually the final TCD value which is the most important or the difference in TCD value from pre-chemotherapy to after chemotherapy which holds the most prognostic significance.

5. TUMOUR INFILTRATING LEUCOCYTES AND RELATIONSHIP WITH PROGNOSIS IN OESOPHAGEAL CANCER

5.1 Background- human immunology

The primarily role of the human immune system is to protect the host from diseases secondary to bacteria, viruses or other pathogens (346). All organisms, even unicellular ones such as bacteria, are thought to possess some form of immune system. During evolution, humans have developed a sophisticated immune response system which has two main branches; innate (non-adaptive) immunity and adaptive immunity (346).

5.1.1 Innate immune system

The innate system is responsible for the first line of immune protection which includes the physical barriers to disease such as skin and mucosal membranes (346). It is also responsible for a non-specific generic response to microorganisms or toxins that breach these barriers. It is triggered by cell injury or damage (such as in inflammation) or when microbes are detected directly through recognition of abnormal components (347). This innate system is also responsible for the recruitment of immune cells to the site of infection/damage through the secretion of cytokines. It is from this initial innate immune response that leukocytes, macrophages and neutrophils cells arise.

5.1.2 Adaptive immune response

This is a more sophisticated immune response, with specific immune reactions and the ability to gain immune memory to ensure that repeat interactions with the same pathogen are resolved more efficiently (346). Unlike the innate system the adaptive system is antigen

specific and reacts to recognition of a ‘non-self’ antigen. It has two branches which often co-exist:

a.) **Humoral adaptive system:** In this system antibodies recognise and bind to antigens through antigen-antibody complex. These complexes are then taken up and broken down by B cells. Following which the B cell can divide and multiply to produce millions of the same antibody into the circulation to identify other specific pathogens which can also be destroyed (346).

b.) **Cell-mediated adaptive immune system:** T cell lymphocytes are responsible for the cellular mediated adaptive immune response. There are two main types: Helper T cells (CD4) and Cytotoxic T cells (CD8). All T lymphocyte cells have T-cell receptors (TCR) complexes, CD3 receptors (used when staining the T cell population in general) plus other specific co-receptors on their surface. For Helper T cells this co-receptor is a CD4 receptor and for Cytotoxic T cells this is a CD8 receptor. However unlike antibodies T cells cannot recognise and bind directly to antigens. They require antigen presenting cells (APCs- typically dendritic or B cells) to first bind and digest the antigen, before presenting peptide chains of the antigen on its surface on what are known as major histocompatibility complex (MHC) molecules (346). On binding to a MHC lymphocytes release cytokines and other stimulatory signals which lead to activation of macrophages and Cytotoxic T cells which can directly attack the pathogen (348). It is thought that cytotoxic T cells are also able to attack tumour cells in the same way and studies have identified the adaptive immune response as having a role in tumour pathology (218, 348, 349).

5.1.3 Cancer Immunology

5.1.3.1 Immune cell infiltration in cancer overview

There are two routes via which immune cells affect cancers: via their circulating presence in the blood and their influence directly at the tumour microenvironment. There is a substantial

body of work looking at the prognostic effect of different levels of circulating immune cells detected in the blood and in particular examining neutrophil to lymphocyte ratios (NLR) in the blood. High NLRs are associated with poor survival in lung (350, 351), breast (352-355), gastric (356-358), colorectal (359-362), prostate (363, 364) and ovarian cancer (365, 366). However this study will only be examining the prognostic influence of different level of immune cells infiltrating the tumour itself.

Epithelial cancers are composed of a mixture of malignant cells and intratumoural stroma (often referred to as tumour microenvironment) which includes fibroblasts, immune cells, lymphatic and blood vessels (367, 368). Variations in the composition of the tumour microenvironment have been linked to differences in patient prognosis in cancers of the breast (369, 370), lung (371), skin (372), prostate (331), colon and rectum(198, 373, 374). The immune system is thought to have several roles in preventing the development of malignant tumours (217, 219, 220). The first role is the recognition and subsequent elimination of viruses that may induce tumour development (e.g. Human papilloma virus in oesophageal or cervical cancers). The second role is the removal of pathogens which may trigger an inflammatory response, therefore creating an environment conducive to tumour growth and thirdly, the immune system is thought to play a direct role in the removal of tumour cells through recognition of tumour antigens.

These tumour infiltrating immune cells, such as T lymphocytes make up an important part of the tumour microenvironment with studies initially in mice showing that T lymphocytes can prevent tumour development and inhibit tumour progression (218, 223). It has been suggested that this effect is mainly related to the activity of cytotoxic T cells (CD8+) which are referred to as the mediators of the anti-tumour response process, with the capability of direct lyses of tumour cells (348). Subsequent studies in humans suggest that high levels of CD8 and CD4 T cells at the tumour microenvironment is associated with a better prognosis in skin melanomas (375), head and neck cancers (376), breast (237), pancreatic (377), colorectal (231, 378-384), urological(257) and gynaecological malignancies (385, 386).

As well as the CD4 and CD8 subsets of T cells, Regulator T cells (Treg cells) have the properties to be involved in the tumour surveillance process. Treg cells play a role in the adaptive immune response by limiting and supressing the immune response (346). They are distinguishable from other T cells by their expression of Forkhead Box Protein P3 (Foxp3). Their role in tumour immunology is unclear with pro-tumour characteristics and anti-tumour characteristics both shown in tumours of different origins. Treg cells are thought to be key suppressors of the anti-tumour response through the suppression of cytotoxic T cells (387, 388). Various studies have shown high infiltration of Treg cells at the tumour microenvironment is associated with poor prognosis in lung (389), breast (390), gastric (391) and ovarian cancers (392). However studies in colon cancer have shown that high levels of Treg cells in the tumour microenvironment is associated with an improved survival (393, 394).

While T cells have been associated with tumour control, other immune cells have been linked with having a “pro-tumour” effect. Macrophages are one of the other major immune cell components in the tumour microenvironment (395), but unlike the T cells, macrophage infiltration has generally been associated with poor prognosis in various cancers (217, 218, 396, 397). Tumour associated macrophages (TAMS) are thought to aid tumour growth due to the action of their secretion of cytokines, growth and angiogenic factors (398, 399). They are part of the initial host response stimulated by malignant cells. The strongest body of evidence of the “pro-tumour” activity of macrophages comes from haematological malignancies such as lymphoma, where multiple studies have shown that high macrophage levels in the blood are related to poor prognosis (234, 400-402). A similar pro-tumour impact of TAMS have also been demonstrated in solid tumours such as lung (225, 403), breast (238, 241), oral squamous cancer (404), renal (405) and colon cancer (406-409). However there have been contrasting results in colorectal cancer studies with some showing an improved survival in tumours with high TAMS (410, 411). These conflicting results maybe a

result of the phenotypic diversity of macrophages with at least two types of macrophage phenotypes recognised in tumour immunology: M1 and M2. While M1 macrophages have a pro-inflammatory affect, M2 macrophages are prone to tissue repair remodelling and immunoregulation (224). Recent studies used immunohistochemistry against CD163 to try to specifically stain for M2 macrophages which are thought to be the subset of macrophages responsible for tumour progression (408, 411) and recent clinical studies have looked at ways to “polarise” macrophages from their pro-tumour to anti-tumour state as a method of cancer treatment (412-414).

Neutrophils are the most commonly found leukocyte subtype at human tumour microenvironments (245). Like macrophages, neutrophils appear to have tumour promoting activity through promoting angiogenesis and facilitating tumour migration (244, 415-417). While the first paper investigating the impact on cancer survival of high levels of circulating neutrophils in peripheral blood was published in 1970 (418), the first study looking at neutrophils at the actual tumour microenvironment was published over 40 years later in 2006 (419). As with macrophages, a high number of tumour infiltrating neutrophils detected by CD66b immunohistochemistry stain has been related to a poor prognosis in multiple malignancies including melanomas (420), colorectal (421, 422), renal (247, 419), liver (246), gastric (423), cervical (243) and lung cancers (424). A meta-analysis study into tumour infiltrating neutrophils (TINs) in 2014 pooled 3946 patients from 20 studies involving lung, gastric, hepatobiliary, renal, colorectal, cervical and head and neck cancer (242). 17 studies measured the neutrophil levels using CD66b immunohistochemistry and 3 used H&E stained slides. The scoring of the neutrophil infiltration was dichotomized into high and low levels (242). The overall conclusion was that high levels of TINs are associated with poorer cancer specific and overall survival (242). No OeC studies were part of this meta-analysis because at the time no study looking specifically at TINs in oesophageal cancer was published.

There is evidence that a high neutrophil to lymphocyte ratio measured in the pre-operative blood is associated with poor prognosis in a number of cancers (354, 357, 362, 363, 425, 426). However whether this systemic ratio established in circulating blood is reflected in the actual tumour microenvironment remains unclear. It is thought that cancer cells do recruit circulating inflammatory cells to the local environment after the recognition of tumour antigens (217, 218). There is little evidence about the prognostic value of the neutrophil to lymphocyte ratio in the tumour microenvironment. The neutrophil to lymphocyte ratio has been measured in the microenvironment of non-small cell lung cancer and hepatocellular cancer previously and a high lymphocyte to neutrophil ratio has been associated with better disease free and overall survival (246, 424).

5.1.3.2 Oesophageal cancer and immune cell infiltration

The prognostic value of tumour infiltrating T cells (CD3 CD8 or Treg) has been investigated in various studies to date in oesophageal squamous cell carcinomas (248, 251-253, 255, 256), oesophageal adenocarcinoma (249, 254) and mixed histological oesophageal cancer cohorts (250) (Table 25). The majority of studies were done using patients who had undergone no neoadjuvant therapy except for the studies by Zingg et al (248, 249) and Tsuchikawa et al (256), which all had mixed cohorts. The study by Zingg et al included 24 patients with oesophageal adenocarcinomas who were treated by neoadjuvant radiochemotherapy (RCT) followed by surgery and looked specifically at Treg infiltration (249). The amount of positive Treg cells was manually scored and compared between periphery and the central tumour regions. Levels were higher at the periphery of the tumour irrespective of patient treatment modality. No association between infiltration and survival was seen in either of the treatment groups (p=0.156). The other study by Zingg et al (248) involved a small cohort of squamous cell carcinomas (n=49) of which 42.9% received neoadjuvant radiochemotherapy. This study used immunohistochemistry to measure CD3, CD4, CD8 and FoxP3 staining of T cells. Manually counting of immunostained cells at the peripheral as well as central region of the tumour was performed. Cut-offs were made

between high and low groups using the median for each stain. In patients who had undergone neoadjuvant therapy, there was a significantly higher level of CD3 and CD4 in the tumour periphery. However, there was no relationship between T cell subtype and overall survival. The study by Tsuchikawa included only 18 patients all of which had been diagnosed with squamous cell carcinoma of the oesophagus (256). 8 patients underwent neoadjuvant chemotherapy (44.4%) and in these patients tumour microenvironment there were significantly higher amounts of CD4 and CD8 stained immune cells. No survival benefit was shown in the neoadjuvant chemotherapy patients compared to the surgery alone group ($p=0.701$). However these previous studies relied on subjective selection of where the periphery of the tumour boundaries began and where the areas of highest density in each region were found. The scoring processes relied on manual scoring of immunohistochemistry staining which was time consuming and with the scorers not blinded could have been liable to bias. The studies also had two treatment groups with unbalanced patient groups with regards cancer staging, with the neoadjuvant groups having significantly higher stages of disease. The survival data also showed that both study cohorts found no difference in 3 and 5 year survival between patients who underwent neoadjuvant RCT and those who proceeded to surgery alone. The finding that infiltrating T cells have varying subtype distribution and positions of their highest infiltration are supported by other studies (248, 249, 256) however no study has yet to show a “best” point for scoring.

The only previous tumour infiltrating T cell study to involve an automatic scoring system was carried out by Rauser et al (254). This study involved 118 patients with adenocarcinoma of the oesophagus, all of which had proceeded straight to surgery. Resection specimens were stained with CD3 and CD8 for the detection of tumour infiltrating T cells and cytotoxic T cells. Automated image analysis was used to score the levels of positive staining as a percentage of the total area of the respective tissue microarray (427). ROC curve analysis was performed to established high and low cut offs for each immunohistochemistry stain. A

significant improved overall survival for patients in the high group of CD3 infiltration was identified (Median survival high CD3 30.6 months vs low 21.4 months; p=0.014). Although there was no statistically significant difference in survival between the CD8 groups (0.396), patients in the high CD8 group were noted to have improved survival compared to patients with low CD8 infiltration (254).

Table 25: Summary of the published literature on T cell infiltration in oesophageal cancer.

Author	Year	Histo+	N	Neo adjuvant	Method	Scoring method	T cell IHC stain	Cut-off
Tahara et al (253)	1990	SCC	15	None	Flow cytometry	Colour scan	CD8 CD4	Unspecified
Schumacher et al (250)	2001	Mixed	70	None	IHC	Manual scoring-periphery and central	CD8	High/low unspecified
Cho et al* (251)	2003	SCC	122	None	IHC	Manual scoring – stromal and tumour	CD4, CD8	Equal groups of 4
Yoshioka et al* (252)	2008	SCC	122	None	IHC	Manual scoring at 5 high density areas	FoxP3	Median split into high/low
Zingg et al (248)	2009	SCC	49	42.9% RCT	IHC	Manual scoring Periphery and central	CD3, CD4, CD8, FoxP3	Median into high/low
Zingg et al (249)	2010	Adeno	130	18.5% RCT	IHC	Manual scoring-periphery and central	FoxP3	Median split into high/low
Rauser et al (254)	2010	Adeno	118	None	IHC	Automatic using % nuclei	CD3 CD8	ROC analysis into high/low
Tsuchikawa et al* (255)	2011	SCC	98	None	IHC	Manual scoring at 5 high density areas	CD4, CD8	Median split into high/low
Tsuchikawa et al* (256)	2012	SCC	18	44.4% Chemo	IHC	Manual scoring at 5 high density areas	CD4, CD8, FoxP3	No groups created

* Same investigatory group; +=Histology, SCC= Squamous cell carcinoma; Adeno= Adenocarcinoma

This method of using tissue microarrays and image analysis has been shown to be effective in previous similar studies on tumour infiltrating immune cells in gastric (428), colorectal (383, 394, 429) and endometrial cancer (430). No study to date has looked at the prognostic impact of T cell subsets infiltration in oesophageal cancer treated with neoadjuvant chemotherapy using automatic scoring techniques.

Studies in OeC have concluded that tumour associated macrophages (TAMs) in the tumour microenvironment may play a role in tumour angiogenesis aiding growth and metastasis (398, 431-434) (Table 26). Three studies all involving squamous cell oesophageal cancer patients who underwent surgery alone found that high infiltration of macrophages were associated with poorer survival (398, 432, 434). One showed a significant difference in 5 year survival between patients with a high and low macrophage count (groups determined by dividing patients by the median) at the tumour microenvironment (24.2% vs 56.9% respectively ;$p<0.005$) (398).

Table 26: Summary of the literature on macrophage infiltration in oesophageal cancer

Author	Year publish	Histo+	N	Neo adjuvant	Method	Scoring method	Stain used	Cut-off
Koide et al (398)	2003	SCC	56	None	IHC	Manual scoring in 5 high density areas	CD68	Median split into high/low
Guo et al (432)	2007	SCC	137	None	IHC	Manual scoring at tumour stroma and tumour centre	CD68	Means used as cut-off into high/low
Dutta et al (431)	2012	Adeno	121	38.8% chemo	IHC	Morphometric scoring	CD68	Median split into high/low
Shigeoka et al (434)	2013	SCC	70	None	IHC	Manual scoring at 3 high density areas	CD68, CD163 and CD204	Median split into high/low
Sugimura et al (433)	2015	SCC	210	49.5% chemo	IHC	Manual scoring at 3 high density areas (vary)	CD68 and CD163	Median split into high/low

+= Histology; SCC= Squamous cell carcinoma; Adeno= Adenocarcinoma

All three studies investigated only patients with oesophageal SCC and all patients were from Asia. All three studies used manual scoring to assess for macrophage infiltration at high density regions. Dutta et al is the only TAM study to date which used quantitative measurements using automated image analysis (431). This UK study included 47 patients (38.8%) who had undergone neoadjuvant chemotherapy. The cohort was a mix of adenocarcinomas (81%) and squamous cell carcinomas (19%), TMAs were stained with CD68 to assess for macrophages and with CD8 for cytotoxic lymphocytes. Morphometric scoring methods were carried out to get a quantitative value of the CD68 and CD8 cell infiltration. It was shown that a high level of macrophage infiltration at the tumour microenvironment was an independent poor prognostic marker (p=0.041). However survival analyses were only performed on the subgroup of adenocarcinomas (due to small numbers in the SCC group) and only from the surgery alone treated cases (n=53). The choice of using the patient who did not undergone chemotherapy for the subgroup analysis was carried out as the investigators were concerned that neoadjuvant chemotherapy would influence the clinicopathological characteristics of the study. This also showed that CD68 infiltration to be an independent prognostic marker in patients who underwent surgery alone (431). Despite the fact it was demonstrated that patients who had undergone chemotherapy had higher infiltration of both CD8 and CD68 no subgroup survival analysis in these patients was carried out. Another criticism of this study was that despite doing staining for both macrophages and CD8 T cells no ratio analysis was carried out.

Sugimura et al compared immune cell infiltration of both macrophages and CD8 T cell in a patient cohort who had neoadjuvant chemotherapy as well as those who had surgery alone treatment (433). This demonstrated that a high infiltration of CD68 positive macrophages was associated with a significantly poorer prognosis but only in patients who had undergone neoadjuvant chemotherapy. Patients with a high infiltration of CD8 had a significantly better

cancer specific survival than patients with low CD8 infiltration (p=0.012). Again no ratio scoring for lymphocyte to macrophages was undertaken.

Two recent studies, one in urological malignancies (257) and one in breast cancer (237) have shown a significant prognostic value in such a lymphocyte to macrophage ratio, although as yet no similar ratio has been assessed in OeC.

The evidence with regard tumour infiltrating neutrophils in oesophageal cancer is currently limited to two studies (Table 27).

Table 27: Summary of literature on neutrophils in oesophageal cancer

Author	Year publish	Histol+	N	Neo adjuvant	Method	Scoring method	Cut-off
Wang et al (435)	2014	SCC	90	None	IHC CD66b	Manual scoring-periphery and central	Median into high/low
Hu et al (436)	2015	Adeno	113	None	IHC CD66b	Manual scoring-periphery and central	Median into high/low

+= Histology

The first by Wang et al involved 90 Chinese squamous cell carcinoma cases, all treated by surgery alone (435). The study used CD66b as a marker for neutrophils and CD8 to identify cytotoxic T cells. Stained tissue samples were independently manually counted by two observers to assess CD66b positive stained cells peripherally and centrally within the residual tumour. This study concluded that a high number of intratumoural neutrophils was associated with the presence of lymph node metastasis, advanced tumour stage and higher recurrence rates (435). A high number of intratumoural neutrophils was also associated with poor disease free and poor overall survival ($p<0.001$ for both). Multivariate cox regression analysis including patient age, gender, tumour location, tumour length, differentiation grade, TNM classification and immunohistochemistry stains identified neutrophil tumour infiltration as an independent prognostic marker, alongside TNM classification. This study measured CD8 positive T cells and calculated the neutrophils to lymphocytes ratio. However, the neutrophil/lymphocyte ratio was not related to prognosis. This study was followed by a

further study looking at surgery alone patients from China but this time involving 112 adenocarcinomas of the oesophagus (436). The study concluded that a high infiltration of neutrophils was associated with a reduced cancer specific survival ($p<0.0001$). No other immune cell staining was carried out in this study. No study to date has examined the difference in neutrophil infiltration in a cohort after being treated with neoadjuvant chemotherapy and neutrophils to lymphocyte ratios in this microenvironment.

This study involving potentially 508 cases from the OE02 trial aiming to examine the prognostic role of leucocytes (CD45), B cells (CD20), T cells (CD3) and T cell subsets (CD8 and Foxp3), macrophages (CD68) and neutrophils (CD66b) in oesophageal cancer patients who had or did not have neoadjuvant chemotherapy. Quantitative scoring was established using automated image analysis (in house development, A Wright) to determine infiltration levels for each immune cell type per case. Differences in the infiltration levels of each immune cell, across the two treatment arms will be assessed as well as the association with clinicopathological variables. Cut-offs will be established to create prognostic groups before survival analysis will determine the prognostic value of each immune cells infiltration. The prognostic value of ratio of T cells to macrophages or neutrophils will also be assessed to see whether this maybe a more powerful prognostic marker than the individual immune cells infiltration levels.

5.2 Methods

5.2.1 Patient cohort

From the OE02 trial (n=802) LICAP received 508 cases resection material for inclusion in this study (3.2.1 OE02 material local collection). Cores were taken of tumour areas and normal tissue from each case. Cases with complete pathological response hence no residual tumour or cases with no evidence of tumour present on any of the material received were excluded.

5.2.2 Tissue microarray (TMA) construction

Tissue microarrays (TMAs) were created and prepared by Mrs L Hewitt, the senior technician of the upper gastrointestinal group at LICAP under the supervision of HG as described previously (437, 438). The TMA maps are shown in Appendix 5: Tumour Micro-Arrays Maps. In total 1112 cores were distributed over 9 TMA maps, which represented the LICAP received cohort. These cores included tumour cores and normal tissue cores. All the cores were stained 7 times using the 7 immunohistochemistry (IHC) stains; CD3, CD8, CD20, CD45, CD66b, CD68 and Foxp3.

5.2.3 Immunohistochemistry technique

The different percentage of the tumour infiltrating immune cells (neutrophils, lymphocytes, macrophages and subtypes as appropriate) were identified using immunohistochemical (IHC) staining techniques. The protocol used for immunohistochemistry is provided in detail in Appendix 4: Detailed immunohistochemistry protocol. In summary, 4 micron sections were cut from each TMA and dried at least overnight. Sections were dewaxed according to a standard protocol and antigen retrieval was performed by microwaving in 10 mmol citrate buffer (pH, 6.0) for 12 minutes. The Dako REAL™ Detection System (Dakocytomation, Glostrup, Denmark, catalogue no. K5001) was used according to the instructions of the manufacturer. Protocols for the individual antibodies including best dilutions of the primary antibodies etc. had been established previously and details of immunohistochemistry staining are shown in Table 28.

Antibodies against CD3, CD8, CD45, CD68 and CD20 were purchased from Dako. Foxp3 and CD66b were purchased from Abcam (Abcam, Cambridge, UK). After incubation with the primary antibody the secondary antibody was applied. ABC complex and DAB solution were applied before slides were stained using Haematoxylin. The slides were the dehydrated and mounted using a DPX medium.

Table 28: Immunohistochemistry details

Target immune cell	Antibody	Catalogue no.	Clone	Dilution	Incubation time
Pan T cells	CD3	M7254	F7.2.38	1:50	Overnight 4 degrees Celsius
Cytotoxic T cells	CD8	M7103	CD8/144B	1:200	Overnight 4 degrees Celsius
Regulator T cells	FoxP3	ab20034	236A/E7	1:50	Overnight 4 degrees Celsius
Pan leukocytes	CD45	M0701	2B11+PD7/26	1:200	Overnight 4 degrees Celsius
Pan macrophages	CD68	M0876	PG-M1	1:200	1 hour at room temperature
Pan neutrophils	CD66b	ab197678	G10F5	1:30	1 hour at room temperature
Pan B cells	CD20	M0755	L26	1:200	1 hour at room temperature

5.2.4 Immune cell infiltration scoring

Each stained TMA slide was scanned at x40 magnification using the Aperio XT slide scanner, (Leica Biosystems, Nussloch, Germany) to create digital slides which were stored on a server with internet access. No demographic or clinicopathological data was stored along with these files. Each slide was reviewed once uploaded to ensure slides were correctly labelled and optimally scanned. These virtual TMA slides were then uploaded onto a different server for automatic image analysis which was performed by AW using in house developed software (439, 440).

5.2.5 Quality control pre-analysis

Before the percentage of positive pixels (POPP) could be established from each core quality control checks were done to ensure POPPs were assigned to the correct case, quality control checks were done. Two observers reviewed each virtual TMA slide to identify any cases or cores that needed to be excluded from final analyses (TS and HG).

1. Each virtual slide image was reviewed to check whether TMA map orientation matched that indicated in the default TMA maps alignment. Any TMA slides not in a matching orientation were adjusted.
2. Next every core was individually reviewed and checked against the original virtual slide image to ensure that cores were in the correct position.
3. Once the observer was happy the correct core was in the correct allocated position the next check was to ensure the core was centred in the snapshot image before analysis.

5.2.6 Automatic scoring of staining

The quality controlled slides where then automatically analysed using the TMAi® software (University of Leeds, UK, http://129.11.65.182/TMAi). The software analysis was run by AW. TMA slides were automatically de-arrayed using an in-house developed computer vision algorithm, and assigned an unique identification number. Once each core had been separated it was processed using another in-house developed algorithm, using colour deconvolution to separate staining channels into Haematoxylin and DAB images, and analyse independently. Colour deconvolution has been shown to provide a robust method of assessing immunohistochemistry staining (441, 442) and has been used previously to quantify components of the tumour microenvironment in ovarian cancer (443). The same technique has also been used to assess the tumour microenvironment in gastric cancer (438). The intensity of DAB staining was co-localised with a basic binary mask of foreground tissue (generated by thresholding of hue, saturation and intensity channels), in order to obtain the total percentage of positive pixels within the tumour area (Figure 29). The percentage of positive pixels (POPP) was used as surrogate for the quantity of immune-positive cells per core.

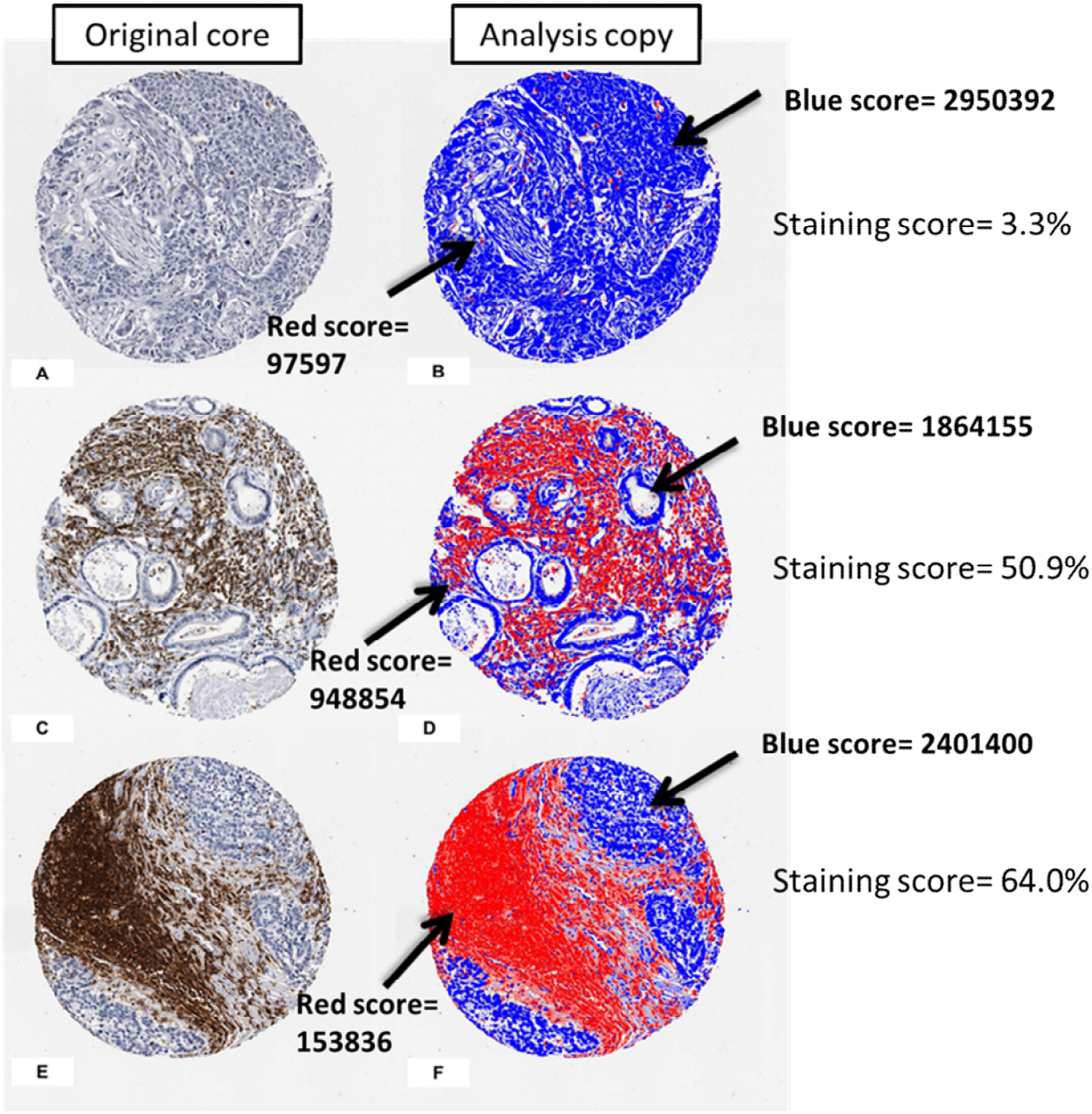


Figure 29: Examples of TMAi colour detection.
3 UGI cases: A+B= UGI0062 with CD68 staining; C+D= UGI2018 with CD3 staining; E+F= UGI0197 with CD45 staining. Each case is shown with their original stained image and their "analysis copy" image where only blue (negative) and red (positive) colours are used to enable a pixel score to be achieved. The red pixels represent the detected and quantified brown (e.g. immuno-positive cells) and the blue pixels all other tissue in the core.

Once an individual TMA slide had been completely analysed the TMAi® program then produced an excel spreadsheet showing the internal departmental case number (UGI number), the respective cores pixel counts for colours represented and the percentage of positive pixels (POPP).

5.2.7 Quality control post analysis

The next stage of quality control was to look at both the original immunostained core and the core created for image analysis (Figure 29) to check the following features:

1) All cores labelled as “tumour” cores did contain viable tumour. Cores without tumour and those with a mixture of tumour and normal epithelium were excluded from analyses
2) All cores used for analyses were free from artefacts like folds, artificial or excessive staining etc.
3) The staining was only present in immune cells. Cores with staining of non-immune cells were excluded
4) The image analysis detected the positive cells with the appropriate threshold

These quality control checks were initially done by TS and then double-checked by HG. In total, 5022 individual tumour cores were reviewed in this final step of quality control.

In situations where there was more than 1 core result for a particular OE02 case the mean POPP was calculated and taken as the final result.

5.2.8 Manual scoring of CD20 and CD66b

After the pre-analysis and post-analysis quality control checks it became evident that the automatic POPP results for cores stained with CD66b were in some cases affected by some light brown background staining. Visually, it was easy to distinguish between the strongly stained neutrophils and the non-specific background. However, attempts to choose different thresholds for the automated image analysis were not successful and thus, the automated analysis would have produced an artificial high value POPP in some cases. Thus, it was decided to score CD66b manually on the original glass slides by two observers (TS and HG)

It was also noted that in some of the cores there was extremely little CD20 staining only representing a few cells. As there was some concern that these small amounts might not be detected if the scanned image was not exactly in focus, and adjustment of the detection

threshold led to an over detection, a decision was made to score CD20 manually on the original glass slides by two observers (TS (all) and double checked by HG or DT). The following method was used for manual scoring for both antibodies;

- Positive= at least one core with evidence of positive staining of immune cells
- Negative= no evidence of staining of immune cells
- Non-informative = cores with technical artefacts which made the core unscorable, missing cores, cores with no tumour.

5.2.9 Statistical analysis

Statistical analysis was performed using the computer program Statistical Package for the Social Sciences (SPSS) 21.0 (Chicago, Illinois). The OE02 trial database was provided by the MRC Clinical Trial Unit at UCL, London, UK. All median ages and percentages (ranges) are given to one decimal place. Mean values of percentage of positive pixels (POPP) per stain were compared between the two treatment arms. Mann Whitney or Kruskal Wallis non-parametric testing was used to assess whether there was a relationship between IHC results and treatment arm, age, histological type, gender, grade of differentiation, T category (TNM6), N category (TNM6), lymphatic or blood vessel involvement and Mandard tumour regression grade. Follow up time was calculated from randomisation to patient death or end of study period. Survival plots were constructed using the Kaplan-Meier method (270) any differences between groups were tested by the log-rank test.

Groupings for initial Kaplan Meier plots were created by dividing the cohort into equal sized groups of 5, 4, 3 and 2. Further modified or simplified groups were created after visual inspection of these initial plots.

CD3:CD68 ratio was created by dividing the CD3 POPP by the matching POPP for CD68 of the same case.

A Cox's proportional hazard model was used for univariate and multivariate analysis. Variables tested in univariate testing were each immune cell POPP initially. Only variables that were significant in univariate survival analysis were included in multivariate analysis against established prognostic clinico-pathological variables from work done earlier (3.3.4 Survival analysis). A p-value of less than 0.05 was considered to be significant.

5.3 Results

5.3.1 General cohort descriptive results

The clinicopathological variables distribution of patients which were included in the TMA study is shown in Table 29. All originated from OE02 trial cases received by LICAP. The median (range) age was 62.6 years (30-83.2 years), 77.9% of patients were male and. 201 (46.9%) were treated with neoadjuvant chemotherapy followed by resection. T0 category cases (i.e. complete responders) were not included as there was no residual tumour per definition. This is led to the greater percentage of surgery alone patients in this cohort.

Median (range) follow up time was 1.46 years (0.02 to 13.21 years) with 73 patients alive at the end of follow up (17%). The median follow up time from randomisation in the patients still alive at the end of trial period was 5.59 years (range 0.16 to 13.21 years). 283 patients died due to a cancer related cause (66%).

Table 29: Clinicopathological characteristic of the IHC cohort

	IHC cohort n=429	
	number	%
Gender		
Male	334	77.9
Female	95	22.1
Treatment arm		
Chemotherapy+ surgery	201	46.9
Surgery alone	228	53.1
Histology		
Adenocarcinoma	313	73.0
Squamous	104	24.2
Other	12	2.8
No residual	0	0.0
Unknown	0	0.0
Differentiation		
Poor	188	43.8
Moderate	203	47.3
Well	34	7.9
Unknown	4	0.9
No residual	0	0.0
T category		
T0 (no residual)	0	0.0
T1	37	8.6
T2	42	9.8
T3	345	80.4
T4	5	1.2
N category		
N0	153	35.7
N1	276	64.3
Lymphatic invasion		
Yes	152	35.4
No	242	56.4
Suspicious	34	7.9
Unknown	1	0.2
Vascular invasion		
Yes	57	13.3
No	337	78.6
Suspicious	35	8.2
Unknown	0	0.0
Mandard grade*		
TRG1	0	0.0
TRG2	2	0.5
TRG3	31	7.2
TRG4	156	36.4
TRG5	239	55.7
Unknown	1	0.2

* Mandard grading; TRG 1= complete regression (excluded), TRG2= only a few residual tumour cells evident, TRG3= predominantly fibrosis, TRG4= predominantly tumour cells and TRG5= no evidence of any regression seen. Median ages and all percentages are given to 1 significant figure, with TNM6 classification used for T and N staging.

Table 30 summaries the number of OE02 cases included for analyses from each IHC stain, with median 337 cases per stain (range 307 to 352 cores). The median drop out percentage for each stain was 20.9% (range: 17.9-23.5%).

Table 30: The number of cores and cases available for analyses per immunohistochemistry stains

Stain	(target cells)*	Total tumour cores	No. of unique OE02 cases	No cases excluded (%)	OE02 cases included in analysis
CD45	(pan leucocytes)	558	429	77 (17.9%)	352
CD3	(pan T cells)	558	429	101 (23.5%)	328
CD8	(cytotoxic T cells)	558	429	92 (21.4)	337
Foxp3	(Treg Cells)	558	429	87 (20.3%)	342
CD68	(pan Macrophages)	558	429	92 (21.4)	337

* CD20 and CD66b not shown as separately scored

5.3.2 Immune cell distribution in the cohort

The median and ranges of POPP measured for each stain is shown in Table 31. This demonstrates that the median POPP per case is below 5% for all types of T cells and macrophages. As expected, a higher median % is seen for CD45 which stains all leukocytes. The manual scored stains (CD20 and CD66b) are shown in section 5.3.4 . Figure 30 shows representative examples of the IHC using a single OE02 case (UGI2018).

Table 31: Median (range) of POPP for the 5 IHC stains quantified by automated image analysis

Stain	(target cells)	Number of scored cases	Total number of case	Median staining %	Range
CD45	(pan leucocytes)	352	429	19.1	1.1-72.4
CD3	(pan T cells)	328	429	4.4	0.5-50.9
CD8	(cytotoxic T cells)	337	429	2.1	0.3-32.5
Foxp3	(Treg Cells)	342	429	2.5	0.3-6.8
CD68	(pan macrophages)	337	429	2.6	0.3-22.4

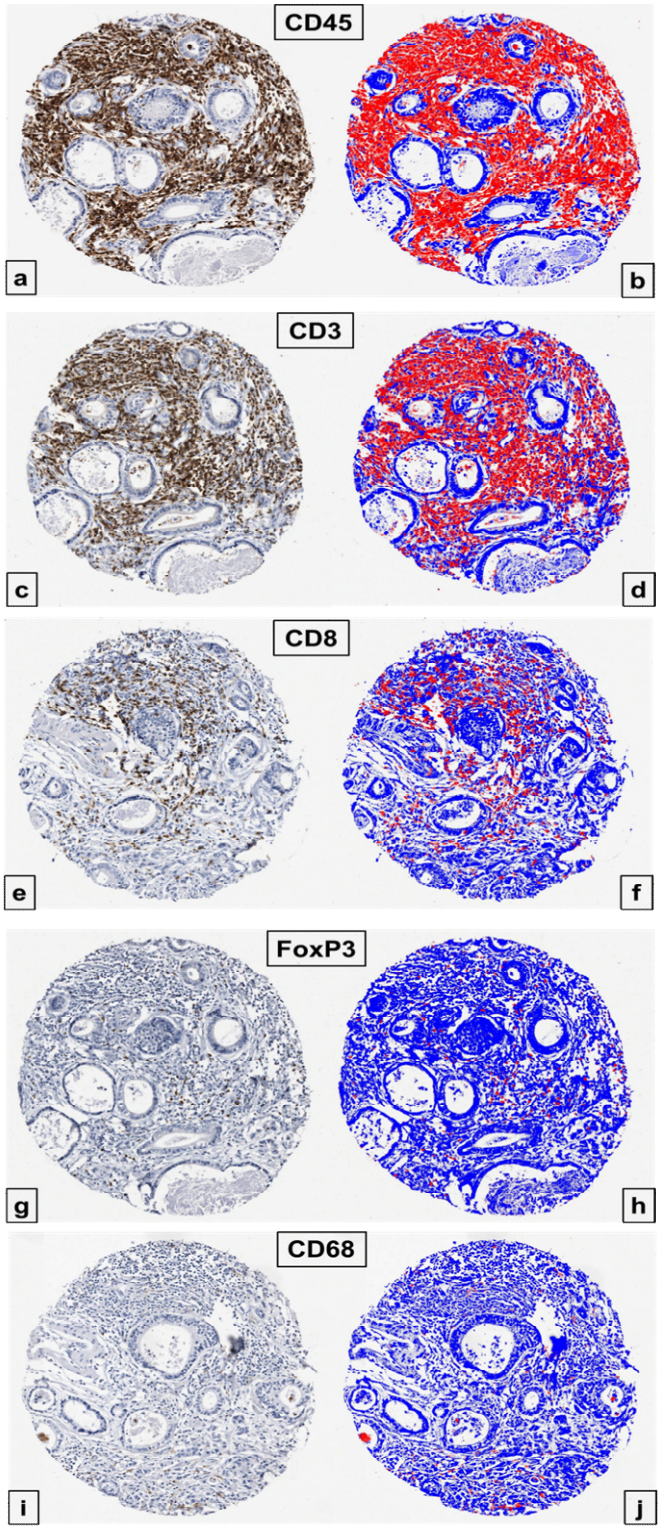


Figure 30(a-h): TMA cores stained with CD45, CD3, CD8, Foxp3 and CD68 from a single OE02 case

The cores shown here are from a single OE02case (cohort number ugi-2018) original cores with brown staining are found to the left and the image analysis output to the right. Although there is evidence of high T cells staining (CD3 images c & d) only a small proportion seem to be regT cells (image g & h) or Cytotoxic T cells (image e & f). This case had a low % of positive macrophage cells (image i & j).

5.3.3 Differences in POPP stain between treatment groups

The differences in the POPP for each IHC stain were examined by stratifying patients according to the two treatment groups. Comparison of the IHC stains between the treatment arms shows no statistical difference for CD3, CD8 and CD45 (Table 32).

Table 32: The relationship between POPP and treatment group

	Chemotherapy and surgery			Surgery alone			
Stain (target cells)	No. cases	Median POPP	Range	No. cases	Median POPP	Range	*p-value
CD45 (pan leucocytes)	159	18.9	1.1-69.4	193	19.9	1.8-72.4	0.319
CD3 (pan T cells)	150	4.4	0.5-36.4	178	4.5	0.7-50.9	0.542
CD8 (cytotoxic T cells)	151	2.1	0.4-20.9	186	2.2	0.3-32.5	0.706
Foxp3 (Treg Cells)	155	2.1	0.3-6.5	187	2.7	0.6-6.8	***<0.001***
CD68 (pan macrophages)	156	2.3	0.4-16.5	181	3.0	0.4-22.4	***0.004***

However, there is a significant difference in IHC staining scores between the two treatment groups for CD68 (macrophages; p=0.004) and Foxp3 (regT cells p<0.001). For both stains, there was a higher median POPP in the surgery alone group compared to the chemotherapy followed by surgery group.

Histograms graphs show the different distributions of CD68 (macrophages) and FoxP3 (regulatory T-cells) scores found in each treatment arm (Figure 31 and Figure 32 respectively).

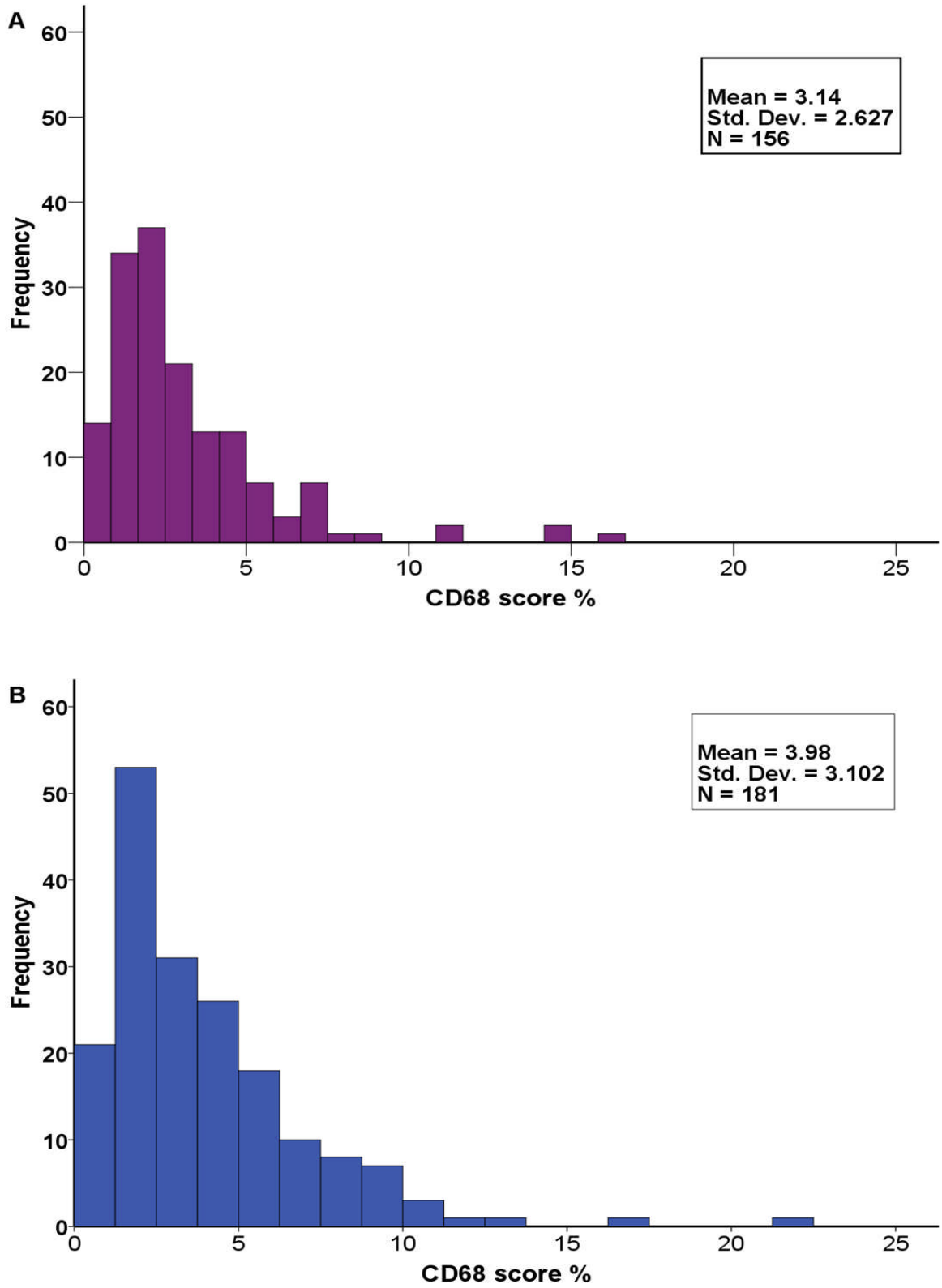


Figure 31: Histogram of CD68 values in (A) chemotherapy + surgery group and (B) surgery alone group.

Distribution of the CD68 (macrophage) staining in the two treatment arms along with the stated mean scores and standard deviation (Std. Dev.). A higher mean macrophage infiltration is seen in the patients who underwent surgery alone (4.04% vs 3.10%, p=0.004).

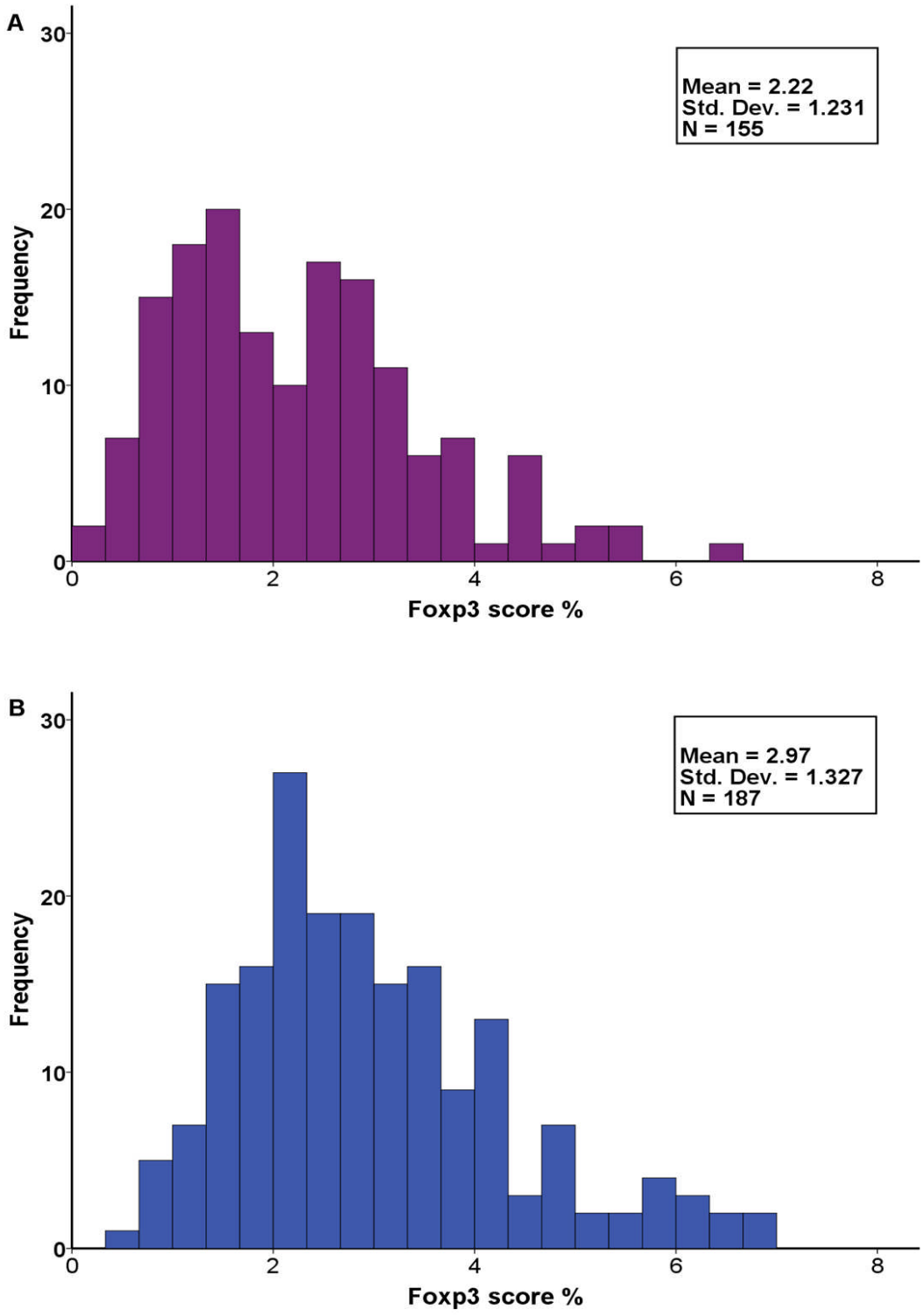


Figure 32: Histogram of Foxp3 values in (A) chemotherapy and surgery group and (B) surgery alone group.

Foxp3 (Regulatory T cells) staining in the two treatment arms along with the stated mean scores and standard deviation (Std. Dev.). A higher mean regulator T cell infiltration is seen in the patients who underwent surgery alone (3% vs 2.22%, $p<0.001$).

5.3.4 Staining results of neutrophils and B cells

The results from the manual scoring of TMA cores stained with CD20 and CD66b is shown below and are divided by treatment arm (Table 33). There was a trend of more positive CD20 staining in the chemotherapy and surgery treatment group which did not reach statistical significance (p=0.060). However the number of CD66b positive cases was significantly higher in the surgery alone group (p=0.034).

Table 33: The CD20 and CD66b manual scoring results by treatment

Stain (n)	Chemotherapy and surgery		Surgery alone		
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	**P value**
CD20 (n=415*)	95 (49.5)	101 (51.5)	86 (39.3)	133 (60.7)	0.060
CD66b (n=352**)	73 (45.5)	91 (55.5)	105 (55.9)	83 (44.1)	***0.034***

*14 CD20 cores deemed unsuitable for assessment or missing
** 77 CD66b cores deemed unsuitable or missing

5.3.5 Relationship between staining results and clinicopathological variables

This initial analysis identified the POPP in the staining of macrophages (CD68), neutrophils (CD66b) and regulatory T cells (FOXp3) was lower in the chemotherapy before surgery treatment group compared to the surgery alone (Table 32).

In the surgery alone treatment group (n=228) there was a significant relationship between CD3 (pan T cells) POPP and tumour histology with adenocarcinomas having higher mean POPP (p=0.009). CD8 (cytotoxic T cells) POPP was significantly higher in adenocarcinomas (p=0.001) and in poorly differentiated cancers (p=0.005). High macrophages (CD68) POPP was more common in adenocarcinomas (p<0.001), poorly differentiated tumours (p=0.003), positive lymph nodes (p=0.004) and those with lymphatic invasion (p=0.031).

CD20 (B cells) and Foxp3 (regT cells) did not have any significant relationship to any of the clinicopathological variables tested (Table 34). The only immune cell POPP shown to have

a significant relationship to T category was CD66b (neutrophils) which had higher values associated with higher T category (p=0.030).

Table 34: The distribution of percentage of positive pixels in surgery alone treatment group (n=228) in relation to clinicopathological variables

	CD3	CD8	CD20	CD45	CD66b	CD68	Foxp3
Age	0.475	0.762	0.074	0.763	0.700	0.496	0.799
Histology	***0.009***	***0.001***	0.334	***0.022***	0.886	***<0.001***	0.070
Grade of differentiation	0.412	***0.005***	0.167	0.245	0.515	***0.003***	0.926
T category	0.433	0.235	0.521	0.605	***0.030***	0.953	0.804
N category	0.117	0.130	0.224	0.107	0.144	***0.004***	0.322
Lymphatic invasion	0.475	0.248	0.373	0.965	0.947	***0.031***	0.336
Blood vessel invasion	0.844	0.699	0.699	0.760	0.638	0.222	0.301
Mandard grade	0.241	0.525	0.198	0.472	0.751	0.275	0.525

In the chemotherapy and surgery treatment group (n=201) the same variables were investigated (Table 35). There was no relationship between CD68 and any of the clinicopathological variables. Similar to the surgery alone group patients, high CD3 (pan T cells) and high CD8 (cytotoxic T cells) was more frequently seen in adenocarcinomas (p= 0.004 and 0.036 respectively).

Table 35: The distribution of percentage of positive pixels in chemotherapy and surgery treatment group (n=201) in relation to clinicopathological variables

	CD3	CD8	CD20	CD45	CD66b	CD68	Foxp3
Age	0.687	0.569	0.874	0.735	0.852	0.164	0.131
Histology	***0.004***	***0.036***	0.071	0.200	0.250	0.094	0.831
Grade of differentiation	0.157	0.414	0.376	0.105	0.184	0.506	0.147
T category	0.098	0.579	0.186	0.083	0.744	0.718	0.876
N category	0.368	0.256	0.355	0.310	0.387	0.641	0.530
Lymphatic invasion*	0.054	0.336	0.276	0.168	0.107	0.702	0.853
Blood vessel invasion	***0.019***	***0.008***	0.470	0.061	0.653	0.086	0.315
Mandard grade	0.739	0.566	0.650	0.497	0.406	0.163	0.054

High levels of CD3 and high levels of CD8 was also related to the absence of blood vessel invasion (p=0.019 and 0.008, respectively).

To determine whether the POPP in the tumour microenvironment is related to prognosis in oesophageal cancer, cox regression analysis was carried out stratified by treatment group.

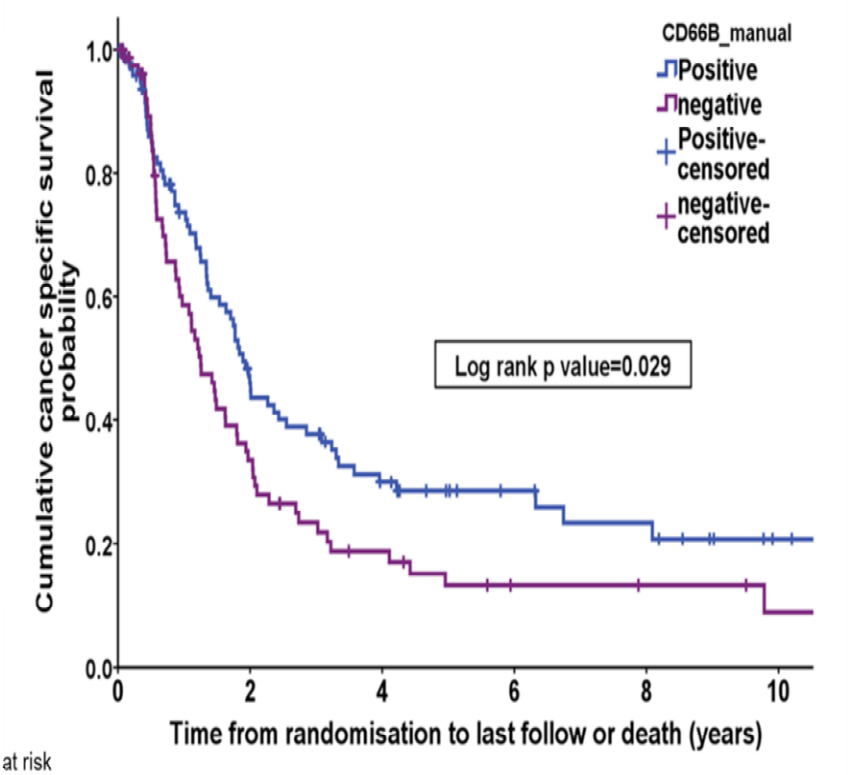
5.3.6 Immune cell infiltration and relationship with survival in surgery alone treated patients

In the surgery alone treatment group (n=228), with the exception of CD66b, none of the other immune cells was related to overall survival in univariate analysis (Table 36). Kaplan Meier plots show a survival benefit for patient with intratumoural CD66b staining as opposed to those tumours who had no evidence of intratumoural CD66b positive cells (Figure 33).

Table 36: Cox regression univariate analysis assessing the prognostic value of the immune cell POPP in the surgery alone treatment group (n= 228).

	Univariate analysis			Univariate analysis		
	OVERALL SURVIVAL			CANCER SPECIFIC survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
CD3	0.98	0.96 to 1.01	0.243	0.99	0.96 to 1.02	0.397
CD8	0.97	0.94 to 1.00	0.065	0.97	0.93 to 1.00	0.072
CD45	0.99	0.98 to 1.00	0.073	0.99	0.98 to 1.00	0.097
CD68	1.00	0.95 to 1.05	0.973	1.03	0.97 to 1.09	0.323
FoxP3	1.03	0.91 to 1.16	0.640	1.07	0.94 to 1.22	0.311
CD20	1.10	0.82 to 1.48	0.535	1.01	0.72 to 1.42	0.961
CD66b	1.33	0.97 to 1.83	0.073	1.48	1.04 to 2.10	***0.029***

HR= hazard ratio, CI= confidence intervals.



Number of patients at risk						
Positive CD66b staining	104	39	22	12	9	2
Negative CD66b staining	82	24	11	5	4	2

Figure 33: Kaplan Meier analysis of cancer specific survival by CD66b status in patients from the surgery alone treatment group.
This survival plot for cancer specific survival in the surgery alone treatment arm shows a significant survival benefit for patients with evidence of neutrophil infiltration (n=105, blue line) compared to those who had no evidence of neutrophil invasion (n=83, purple line) (p=0.029).

To assess whether the presence of CD66b POPP is an independent prognostic marker, a cox regression multivariate analysis was performed including patient age, T category, N category and blood vessel involvement in the model (Table 37). CD66b did not retain a statistically significant prognostic value in multivariate analyses.

Table 37: Multivariate Cox regression analysis including markers prognostic in univariate survival analysis and CD66b POPP

	Multivariate analysis		
	CANCER SPECIFIC SURVIVAL		
	HR	95% CI	*P* value
Age	1.02	1.00 to 1.04	***0.019***
T category	1.51	1.00 to 2.28	***0.048***
N category	1.86	1.22 to 2.84	***0.004***
Blood vessel invasion	1.08	0.84 to 1.38	0.562
CD66b	1.35	0.95 to 1.91	0.097

CI= confidence interval, HR = hazard ratio

5.3.7 Immune cell infiltration and relationship with survival in chemotherapy and surgery treated patients

The same analysis was carried out now looking at the chemotherapy and surgery treatment group. Univariate cox regression analysis showed that higher CD3 staining was related to better overall survival (p=0.043), but no significant relationship to cancer specific survival was demonstrated (p=0.053). No other immune cell score was shown to have any statistical significant relationship with overall or cancer specific survival (Table 38).

Table 38: Univariate Cox regression analysis assessing for prognostic value in the immune cell staining for patients from the chemotherapy and surgery treatment group

	Univariate analysis			Univariate analysis		
	OVERALL SURVIVAL			CANCER SPECIFIC		
	HR	95% CI	*P* value	HR	95% CI	*P* value
CD3	0.96	0.93 to 0.99	***0.043***	0.96	0.92 to 1.00	0.053
CD8	0.95	0.90 to 1.02	0.139	0.96	0.89 to 1.02	0.202
CD45	0.99	0.98 to 1.00	0.080	0.99	0.96 to 1.00	0.149
CD68	1.02	0.97 to 1.08	0.468	1.04	0.98 to 1.10	0.227
FoxP3	0.93	0.81 to 1.07	0.292	0.91	0.78 to 1.06	0.203
CD20	1.19	0.87 to 1.62	0.276	1.28	0.91 to 1.81	0.151
CD66b	0.95	0.68 to 1.33	0.772	0.99	0.68 to 1.43	0.941

CI= confidence interval, HR = hazard ratio

On multivariate cox regression analysis, CD3 did not demonstrate independent prognostic value for overall survival (p=0.195). The ypN category of a patient's OeC was shown to be an independent prognostic marker in this subgroup (Table 39).

Table 39: Multivariate Cox regression analysis assessing the independent prognostic value of CD3 in the chemotherapy and surgery patients

	Multivariate analysis		
	OVERALL SURVIVAL		
	HR	95% CI	*P* value
Age	1.02	1.00 to 1.04	0.111
ypT category	1.31	0.92 to 1.86	0.131
ypN category	1.76	1.19 to 2.61	***0.004***
Blood vessel invasion	1.34	0.99 to 1.81	0.054
CD3	0.98	0.94 to 1.01	0.195

HR=hazard ratio, CI= confidence interval

5.3.8 Developing a quantitative T cell group classification for survival stratification

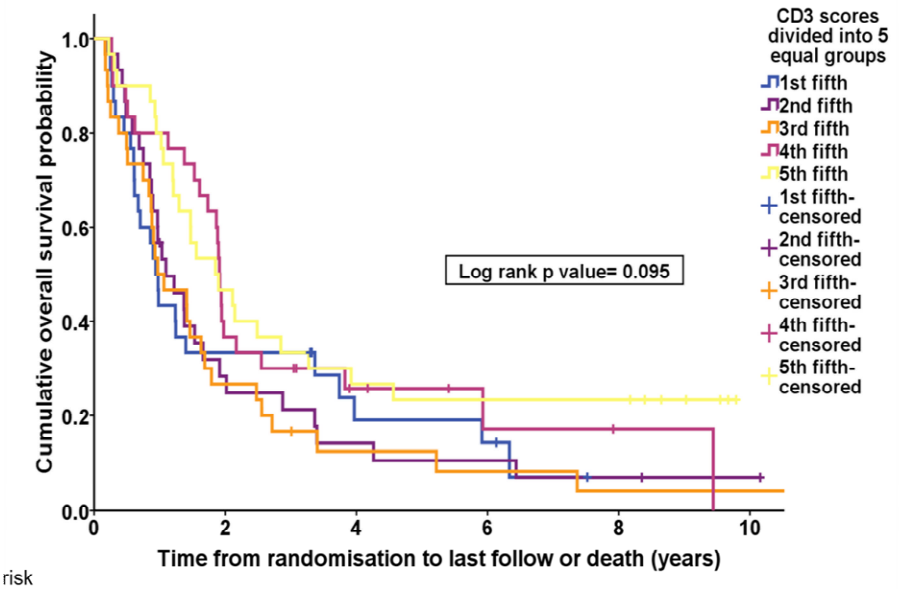
With CD3 showing evidence of prognostic value in patients from the chemotherapy and surgery treatment group (Table 38), CD3 scores were divided into equal groups to establish an optimal cut off. As in the TCD work, CD3 scores were used to divide the cohort into 5, 4, 3 and 2 equal sized groups (Table 40).

Table 40: CD3 percentage ranges when creating equally sized CD3 groups for prognostic stratification

	Chemotherapy and surgery patients (n=150)	
	CD3 range %	No. cases
5 group system		
1st fifth	<2.0	30
2nd fifth	2.0 to 3.4	30
3rd fifth	3.4 to 5.1	30
4th fifth	5.1 to 8.3	30
5th fifth	>8.3	30
4 group system		
1st quartile	<2.5	37
2nd quartile	2.5 to 4.4	38
3rd quartile	4.4 to 7.6	38
4th quartile	>7.6	37
3 group system		
Low third	<3.0	50
Mid- third	3.0 to 6.1	50
High- third	>6.1	50
2 group system		
Low group	<4.4	75
High group	>4.4	75

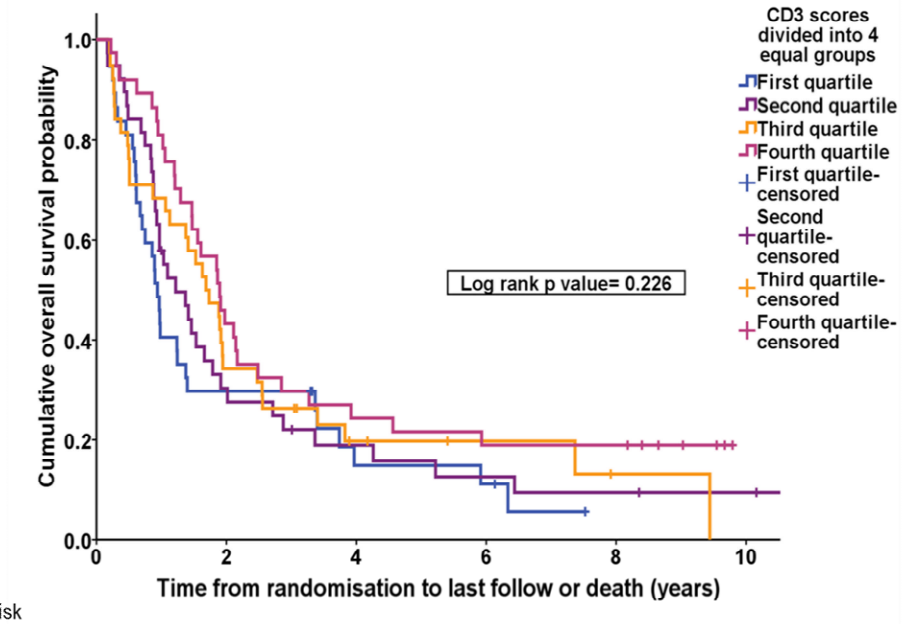
Using these groupings Kaplan Meier survival plots were generated using overall survival data only, as this was where CD3 had shown potential for prognostic influence. Patients with a higher POPP of CD3 staining have a better overall survival than those with a low POPP. These survival plots showed significant difference with overall survival using a 3 group classification (Figure 34 and Figure 35).

a.)



Number of patients at risk						
1st fifth group	30	10	4	3	0	0
2nd fifth group	30	8	4	3	2	1
3rd fifth group	30	8	3	2	1	1
4th fifth group	30	11	5	2	1	0
5th fifth group	30	14	8	7	7	0

b.)

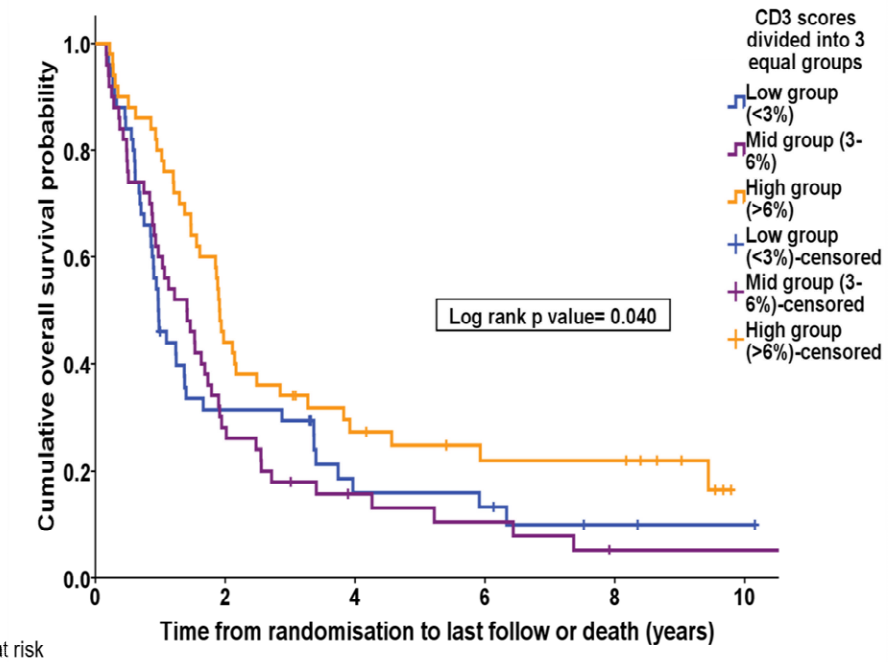


Number of patients at risk						
1st quartile group	37	11	4	3	0	0
2nd quartile group	38	11	6	4	3	2
3rd quartile group	38	13	5	3	1	0
4th quartile group	37	16	9	7	7	0

Figure 34 (a-b): Kaplan Meier survival plots for overall survival in the chemotherapy and surgery treatment group stratified by the 5 and 4 CD3 groupings.

Kaplan Meier survival plots for overall survival in the chemotherapy and surgery treatment group stratified by 5 groups (34A above) and 4 groups (34B below) of CD3 scoring. Neither classification produces a statistically significant difference in overall survival between its groups.

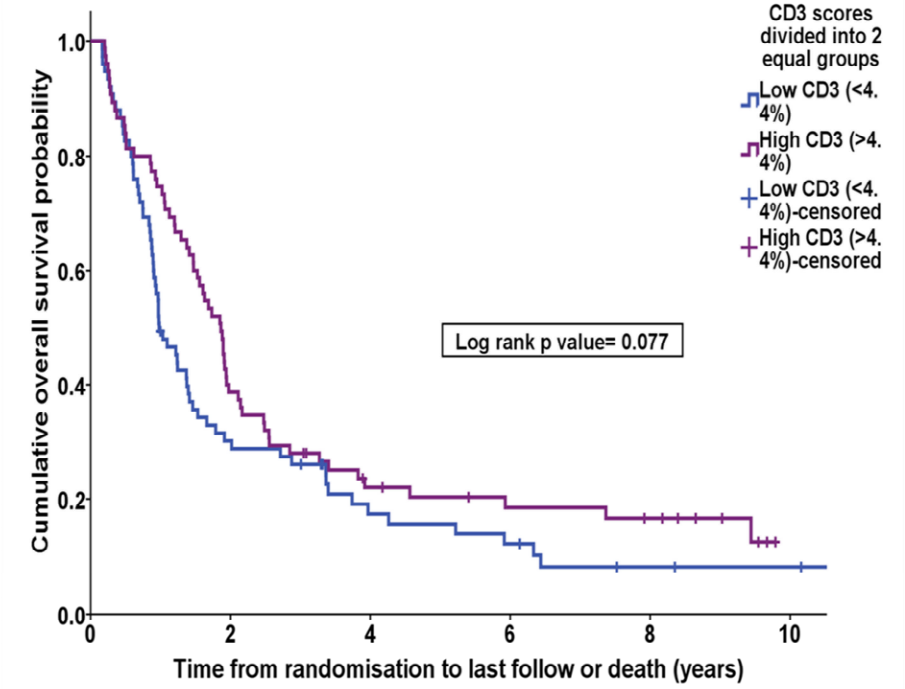
a.)



Number of patients at risk

Low CD3 (<3%) group	50	15	6	5	2	1
Mid CD3 (3-6%) group	50	14	6	4	1	1
High CD3 (>6%) group	50	22	12	8	8	0

b.)



Number of patients at risk

Low CD3 (<4.4%) group	75	22	10	7	3	2
High CD3 (>4.4%) group	75	29	14	10	8	0

Figure 35 (a-b): Kaplan Meier survival plots for overall survival in the chemotherapy followed by surgery patient group stratified by the 3 and 2 CD3 POPP groupings.
Kaplan Meier survival plots for overall survival in the chemotherapy and surgery treatment group stratified by 3 groups (35A above) and 2 groups (35B below) of CD3 scoring. Only the 3 group system gives statistically significant differences in group overall survival (p=0.040).

Examining the 3 group CD3 plot (Figure 35a), the mid and low CD3 group plots are seen to run in close proximity. So to assess whether there would be better patient stratification if the mid and low group were combined and a modified 2 group cut-off was established using a 6% POPP cut-off. This modified 2 group system as opposed to the original 2 group system (by dividing the cohort simple by the median) is shown in Figure 36 and gives much clearer prognostic groups. Log rank calculation show the difference in overall survival between these two modified groups (6% cut-off) is statistically significant (p=0.012).

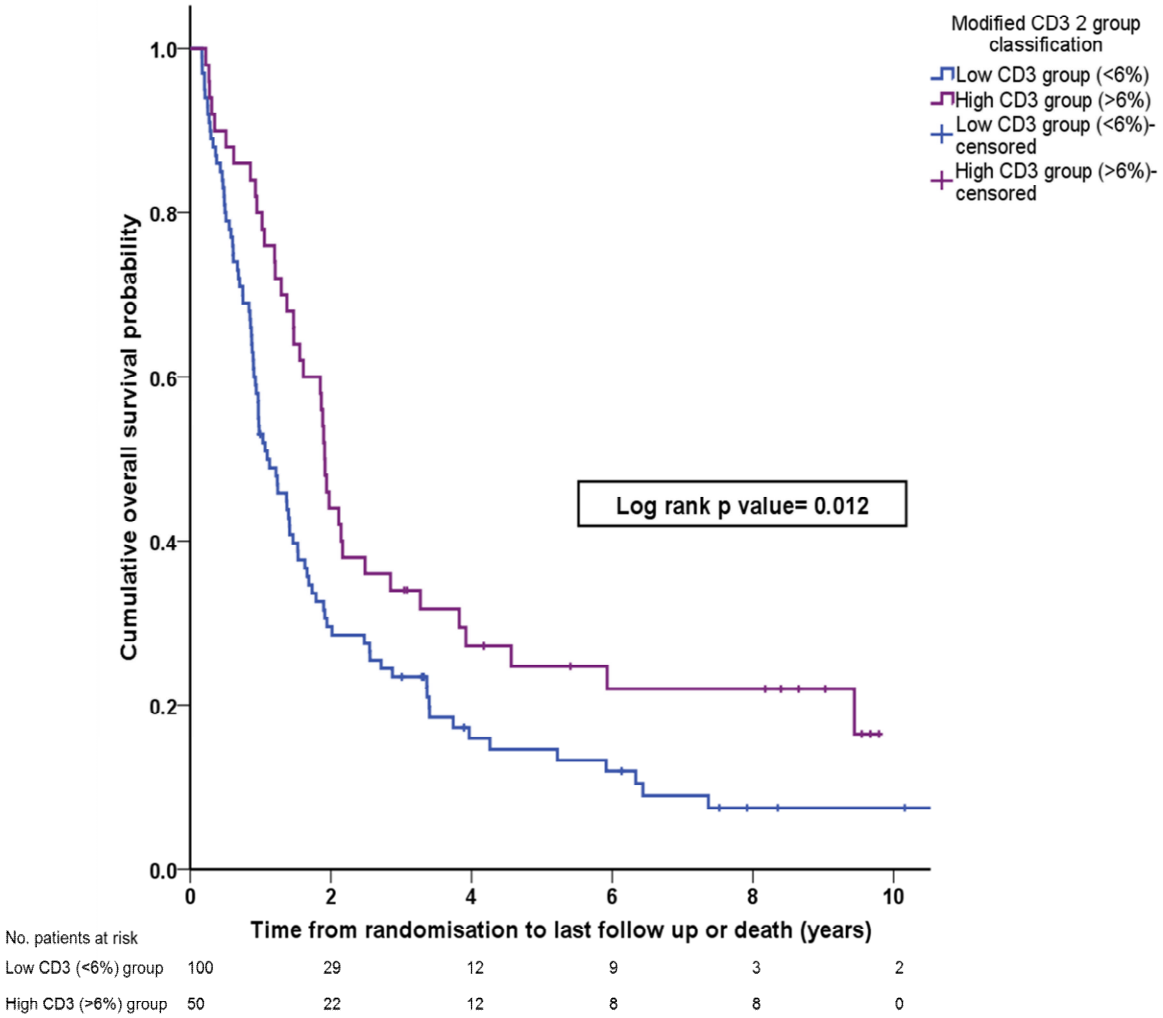


Figure 36: Modified 2 group CD3 classification Kaplan Meier overall survival.
The graph above shows a Kaplan Meier overall survival if patients are stratified into the modified CD3 classifications groups with a statistically significant difference in survival between the two groups.

The differences in the clinicopathological features between the two CD3 groups (using the modified 6% cut off boundary) are shown below in Table 41.

Table 41: Relationship with clinicopathological variables between the modified CD3 two groups in patients from the chemotherapy and surgery treatment group

	Low CD3 group <6% (n=100)	High CD3 group >6% (n=50)	
	Number (%)	Number (%)	P value
Age			
Median	61.4	60.6	0.414
Range	36.4- 77.7	41.6- 83.1	
Histology			
Adenocarcinoma	71 (71.0)	45 (90.0)	
Squamous	27 (27.0)	5 (10.0)	***0.008***
Other	2 (2.0)	0 (0.0)	
Grade of differentiation			
Poor	40 (40.0)	19 (38.0)	
Moderate	50 (50.0)	27 (54.0)	0.887
Well	9(9.0)	3 (6.0)	
Unknown	1 (1.0)	1 (2.0)	
ypT category			
T1	5 (5.0)	5 (10.0)	
T2	9 (9.0)	5 (10.9)	0.654
T3	86 (86.0)	38 (76.0)	
T4	0 (0.0)	2 (0.0)	
ypN category			
N0	31 (31.0)	21 (42.0)	0.184
N1	69 (69.0)	29 (58.0)	
Lymphatic vessel invasion			
Yes	35 (35.0)	10 (20.0)	
No	60 (60.0)	35 (70.0)	0.378
Suspicious	5 (5.0)	5 (10.0)	
Blood vessel invasion			
Yes	10 (10.0)	2 (4.0)	
No	80 (80.0)	47 (94.0)	***0.024***
Suspicious	10 (10.0)	1 (2.0)	
Mandard grade			
TRG1*	0 (0.0)	0 (0.0)	
TRG2	0 (0.0)	1 (2.0)	
TRG3	10 (10.0)	5 (10.0)	0.264
TRG4	36 (36.0)	22 (44.0)	
TRG5	54 (54.0)	22 (44.0)	

* Only cases with residual tumour were included in this immune cell study.

Kruskal Wallis tests identify significant differences in tumour histology (p=0.008) and blood vessel invasion status (p=0.024) between the two CD3 groups (Table 41). Patient age, tumour differentiation, lymphatic invasion status, Mandard tumour regression grade, ypT and ypN categories across the two CD3 groups did not significantly differ.

5.3.9 T cell to macrophage ratio and its potential prognostic value

Given the initial findings of differing roles in OeC prognosis between lymphocytes and macrophages an immune cell ratio for T cells (CD3) to macrophages (CD68) levels was calculated. The CD3 POPP was divided by the CD68 POPP in a total number of 134 (66.7%) cases with matched values in the chemotherapy and surgery treatment group and in 161(70.2%) cases with matched values in the surgery alone group. Table 42 shows the median and ranges for the CD3:CD68 (T cell: macrophages) ratio. This demonstrates a median ratio of 1.74 in the chemotherapy and surgery treatment group, suggesting overall greater infiltration of T cells compared to macrophages. This is compared to the surgery alone treatment group where although there remains a predominance of CD3 cells the median is lower 1.34 (range 0.11 to 16.3).

Table 42: Difference in the T cell to macrophage ratios between the two treatment groups

	Number of cases matched	Median	Range
CD3:CD68 ratio (Chemotherapy surgery treatment group)	134	1.74	0.11 to 24.21
CD3:CD68 ratio (Surgery alone treatment group)	161	1.34	0.11 to 16.3

Table 43 shows that there was a significant relationship between CD3:CD68 ratio and differentiation, ypT category, ypN category and lymphatic invasion status. A higher CD3:CD68 in the chemotherapy and surgery treatment group was more frequent in moderately differentiated tumours, low ypT, ypN0 category tumours with no evidence of lymphatic invasion.

No significant relationship between any clinicopathological variables and CD3:CD68 ratio is seen in the surgery alone treatment group (Table 43).

Table 43: Relationship between CD3:CD68 ratio and clinicopathological variables by treatment arm

	Chemotherapy and surgery n=134	Surgery alone n=161
Age	0.124	0.245
Histology	0.236	0.690
Grade of differentiation	***0.038***	0.065
T category	***0.040***	0.371
N category	***0.037***	0.863
Lymphatic invasion	***0.028***	0.082
Blood vessel invasion	0.222	0.500
Mandard grade	0.135	0.473

The prognostic value of the T lymphocyte to macrophage (CD3:CD68) ratio was then assessed in a univariate cox regression analysis stratifying by treatment groups. In the surgery alone treatment group (n=161) CD3:CD68 ratio had no significant prognostic value in either overall or cancer specific survival (Table 44).

Table 44: Univariate cox regression analysis assessing the prognostic value of the CD3:CD68 ratio in patients from the surgery alone treatment group.

	Univariate analysis			Univariate analysis		
	OVERALL SURVIVAL			CANCER SPECIFIC SURVIVAL		
	HR	95% CI	*P* value	HR	95% CI	*P* value
CD3:CD68 ratio	0.96	0.88 to 1.04	0.315	0.93	0.83 to 1.03	0.159

HR=hazard ratio, CI= confidence intervals

In the chemotherapy and surgery treatment group univariate cox regression analysis showed the CD3:CD68 ratio to have a significant prognostic value ($p<0.001$) for both overall and cancer specific survival. These results suggest patients having a high ratio (i.e. prevalence of CD3 over CD68) have improved overall and cancer specific survival (Table 45).

Table 45: Univariate cox regression analysis assessing the prognostic value of the CD3:CD68 ratio in patients from the chemotherapy and surgery treatment group.

	Univariate analysis			Univariate analysis		
	OVERALL SURVIVAL			CANCER SPECIFIC SURVIVAL		
	HR	95% CI	*P* value	HR	95% CI	*P* value
CD3:CD68 ratio	0.86	0.79 to 0.95	***0.001***	0.83	0.75 to 0.92	***0.001***

HR=hazard ratio, CI= confidence intervals

As CD3:CD68 ratio was identified as having prognostic value for overall and cancer specific survival this was assessed against other established prognostic markers in this treatment group. Therefore multivariate analysis was performed to assess the prognostic value of CD3:CD68 ratio (as a continuous variable) along with patient age, ypT category, ypN category and blood vessel involvement. This demonstrates that the immune cell ratio CD3:CD68 retained its prognostic value in multivariate analysis for both overall and cancer specific survival (p=0.009 and 0.006 respectively). Only CD3:CD68 ratio and ypN category were shown to be independent prognostic markers for both overall and cancer specific survival in tumours in this treatment group (Table 46). The ypT category had independent prognostic value in cancer specific survival analysis only (p=0.046).

Table 46: Multivariate cox regression survival analysis including patient age, ypT, ypN categories, blood vessel invasion and the CD3:CD68 ratio

	Multivariate analysis			Multivariate analysis		
	OVERALL SURVIVAL			CANCER SPECIFIC SURVIVAL		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.01	0.99 to 1.04	0.269	1.01	0.99 to 1.04	0.354
ypT category	1.40	0.94 to 2.07	0.099	1.69	1.01 to 2.81	***0.046***
ypN category	1.96	1.28 to 3.00	***0.002***	2.25	1.38 to 3.68	***0.001***
Blood vessel invasion	1.31	0.96 to 1.81	0.094	1.31	0.92 to 1.86	0.136
CD3:CD68 ratio	0.88	0.80 to 0.97	***0.009***	0.85	0.76 to 0.96	***0.006***

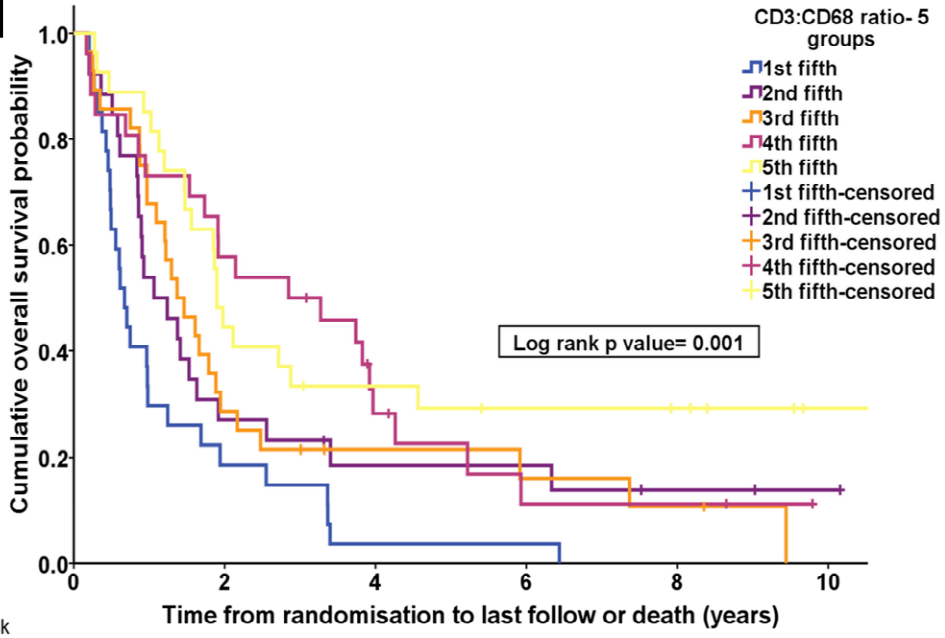
With the CD3:CD68 (T cells to macrophage) ratio being an independent prognostic marker in the chemotherapy and surgery treatment, cut-offs were established using quantitative measures. The chemotherapy and surgery treatment group was divided by each cases immune cell ratio score into 5, 4, 3 and 2 equal groups to produce evenly sized patient groups (Table 47).

Table 47: The chemotherapy and surgery treatment group divided into equal sized groups by CD3:CD68 ratio

Cohort split	CD3:CD68 (n=134)	
	*Cut-off %	Number
5 group		
1st fifth	<0.85	27
2nd fifth	0.86-1.37	26
3rd fifth	1.38-2.17	28
4th fifth	2.18-3.98	26
5th fifth	>3.98	27
4 group		
1st Quartile	<0.97	33
2nd Quartile	0.98-1.75	34
3rd Quartile	1.76-3.29	34
4th Quartile	>3.29	33
3 group		
Low third	<1.14	45
Mid third	1.15-2.47	44
High third	>2.47	45
2 group		
Low group	<1.75	67
High group	>1.75	67

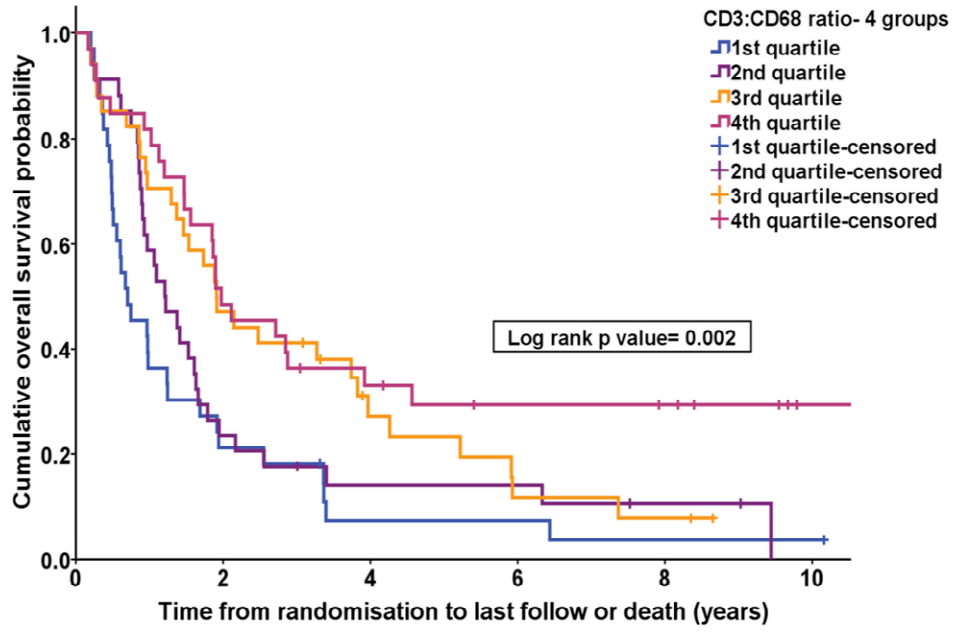
Kaplan Meier survival plots were constructed using these group classifications to determine the classification which visually as well as statistically differentiated overall and cancer specific survival in the chemotherapy surgery treatment group (Figure 37 and Figure 38). These Kaplan Meier plots illustrate that all the classification systems tested did visually stratify both overall and cancer specific survival in the chemotherapy and surgery treatment group. All log rank tests identified significant differences in survival with all the classification systems tested. While the 2 group classification showed the clearest visual survival benefit, the 3 group classification also had a clear survival between the patients in the high ratio group (>2.5) and patients in the mid and low group (Figure 37c and Figure 38c).

a



Number of patients at risk						
1st fifth group	27	5	1	1	0	0
2nd fifth group	26	7	4	4	2	1
3rd fifth group	28	8	4	3	2	0
4th fifth group	26	15	6	2	2	0
5th fifth group	27	12	8	6	5	1

b



Number of patients at risk						
1st quartile group	33	7	2	2	1	1
2nd quartile group	34	8	4	4	2	0
3rd quartile group	34	16	7	3	2	0
4th quartile group	33	16	10	7	6	1

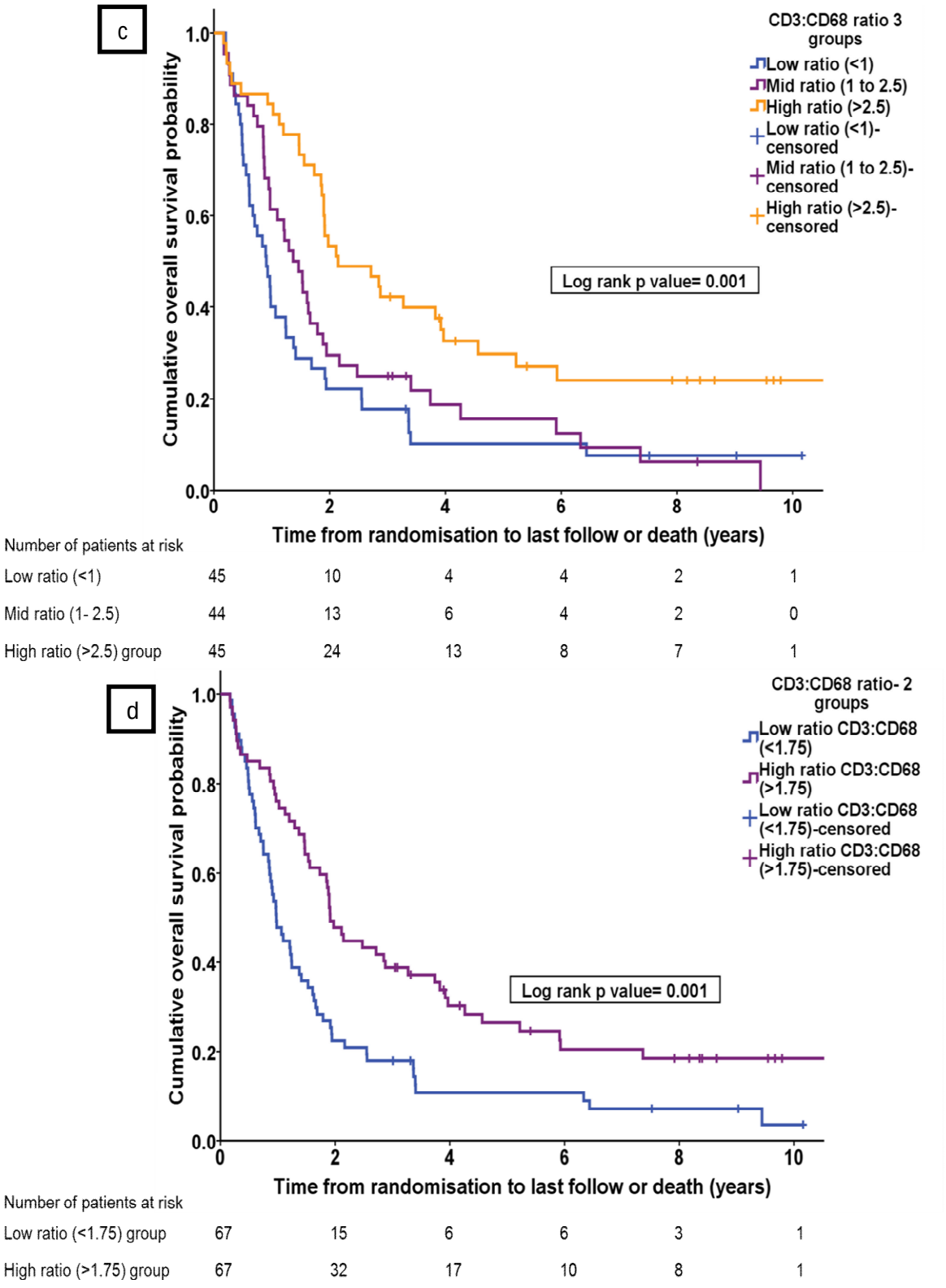
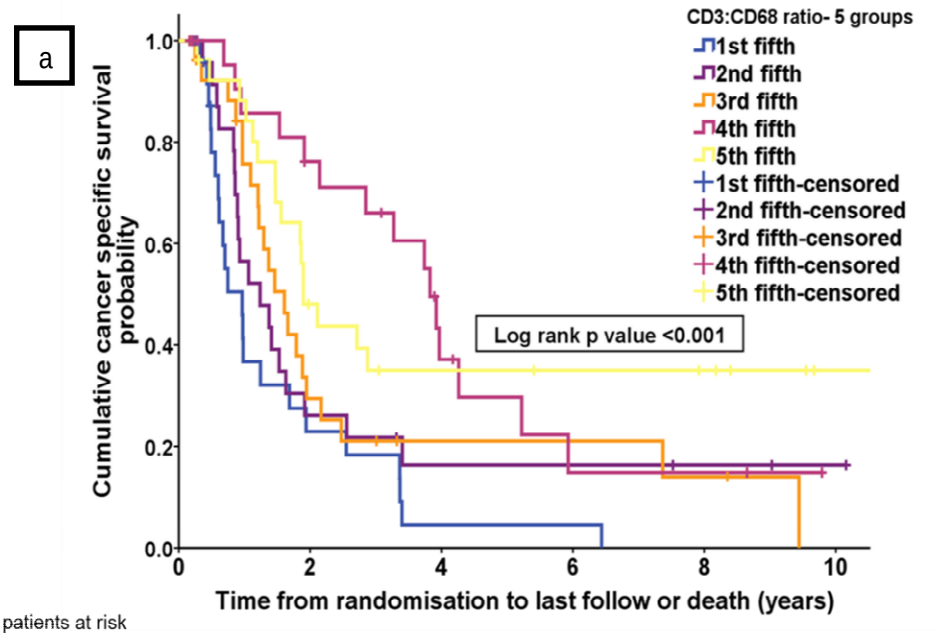
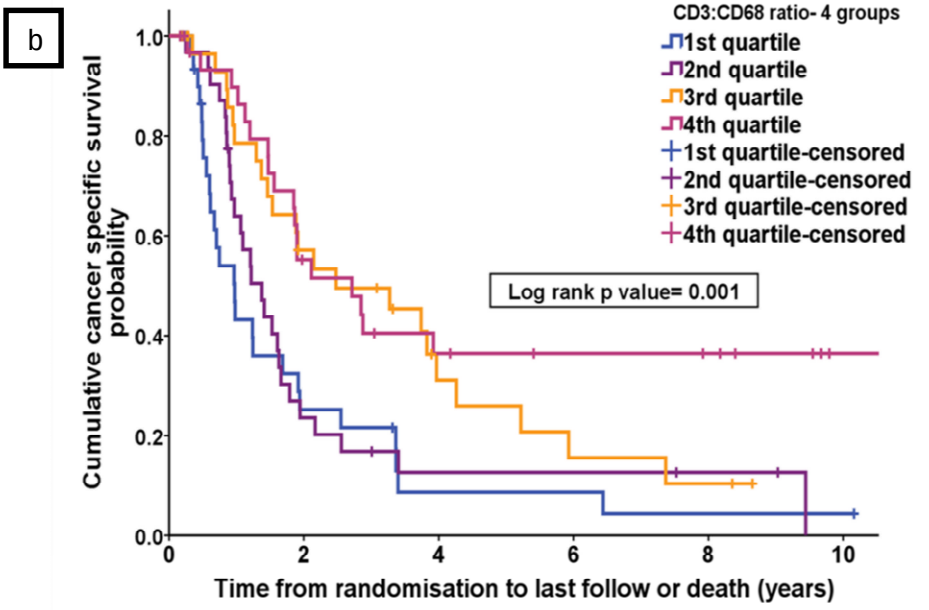


Figure 37(a-d): Kaplan Meier survival plots of overall survival stratified by CD3 to CD68 classification systems (a= 5 groups, b= 4 groups c= 3 groups and d= 2 groups).
The four Kaplan Meier survival plots starting with the 5 group classification (top) to the 2 group classification (bottom). The plots show all 4 systems have significant differences in survival using log rank testing. Each plot demonstrates patients with a high ratio of T cells to macrophages have a statistically significant improved survival overall.



Number of patients at risk

1st fifth group	27	5	1	1	0	0
2nd fifth group	25	6	3	3	2	1
3rd fifth group	27	7	3	3	2	0
4th fifth group	25	15	6	2	2	0
5th fifth group	26	11	7	6	5	1



Number of patients at risk

1st quartile group	33	7	2	2	1	1
2nd quartile group	33	7	3	3	2	0
3rd quartile group	32	15	6	3	2	0
4th quartile group	32	15	8	7	6	1

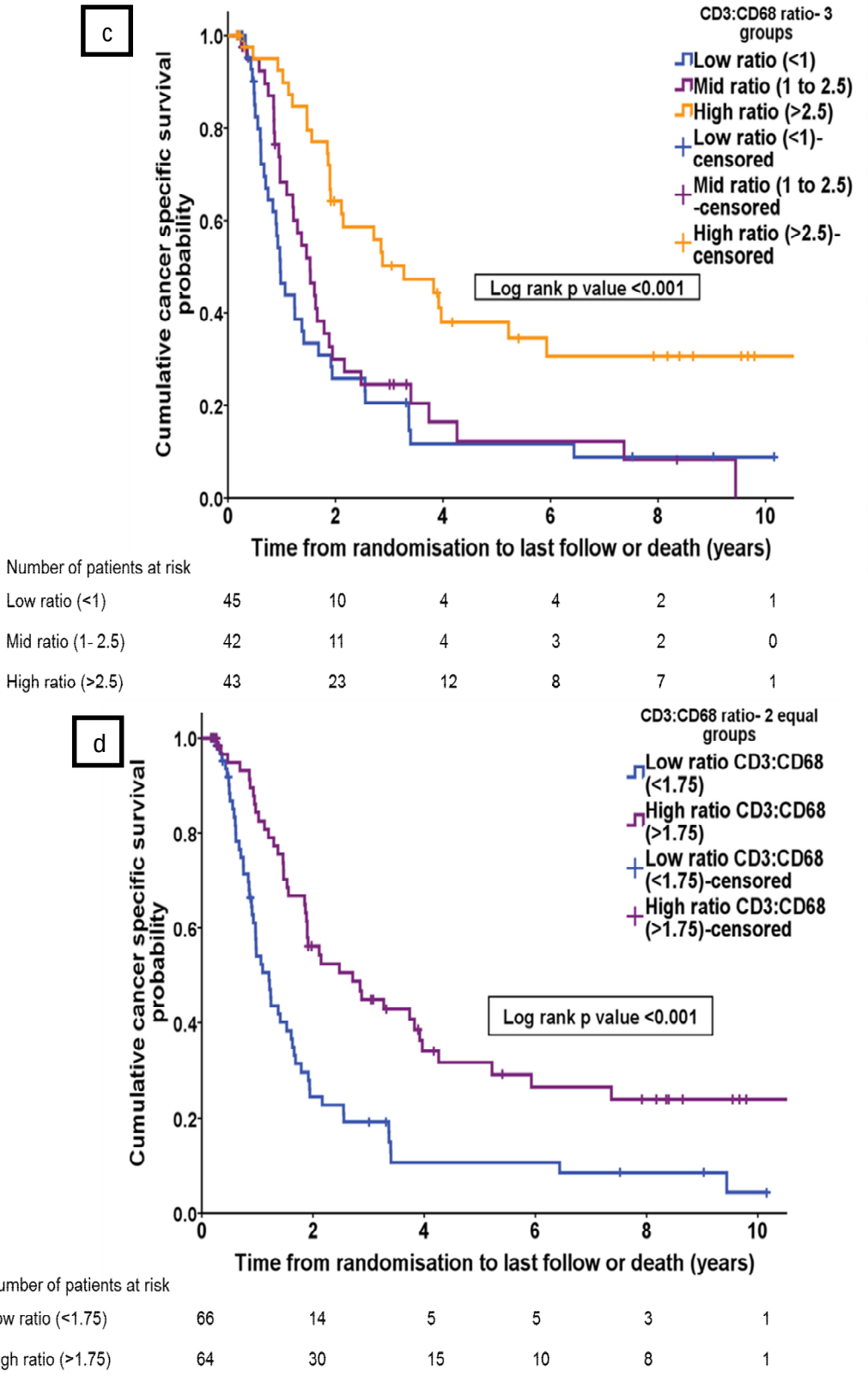
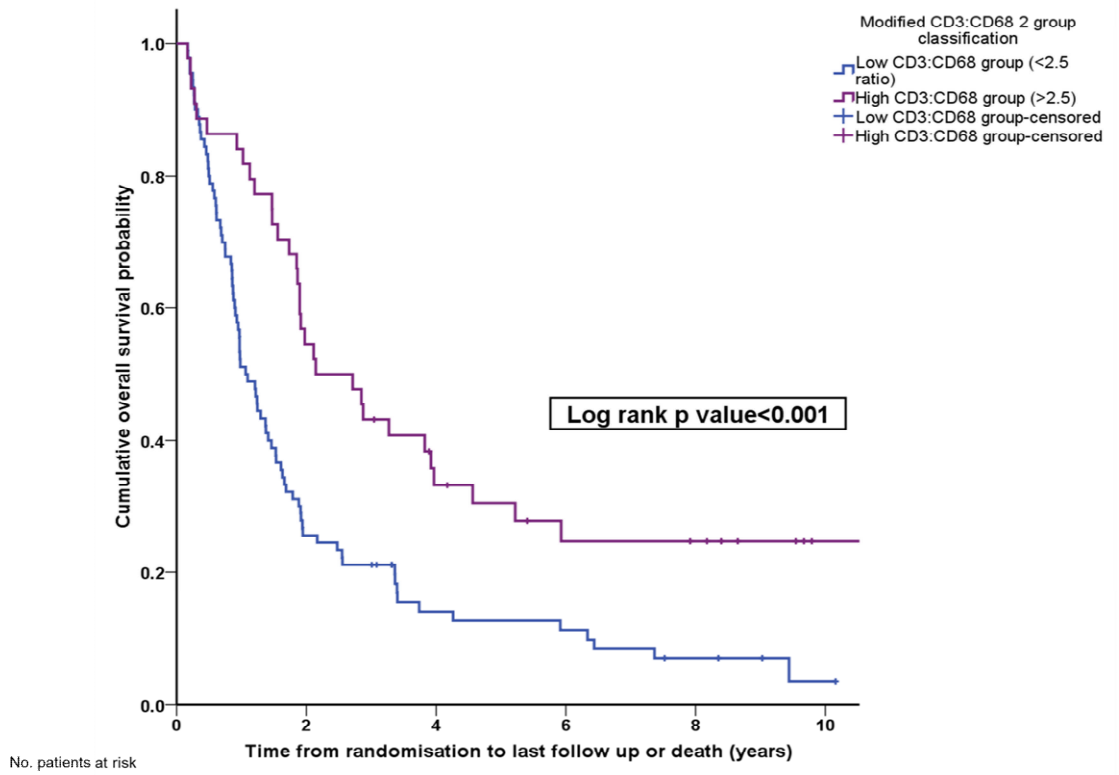


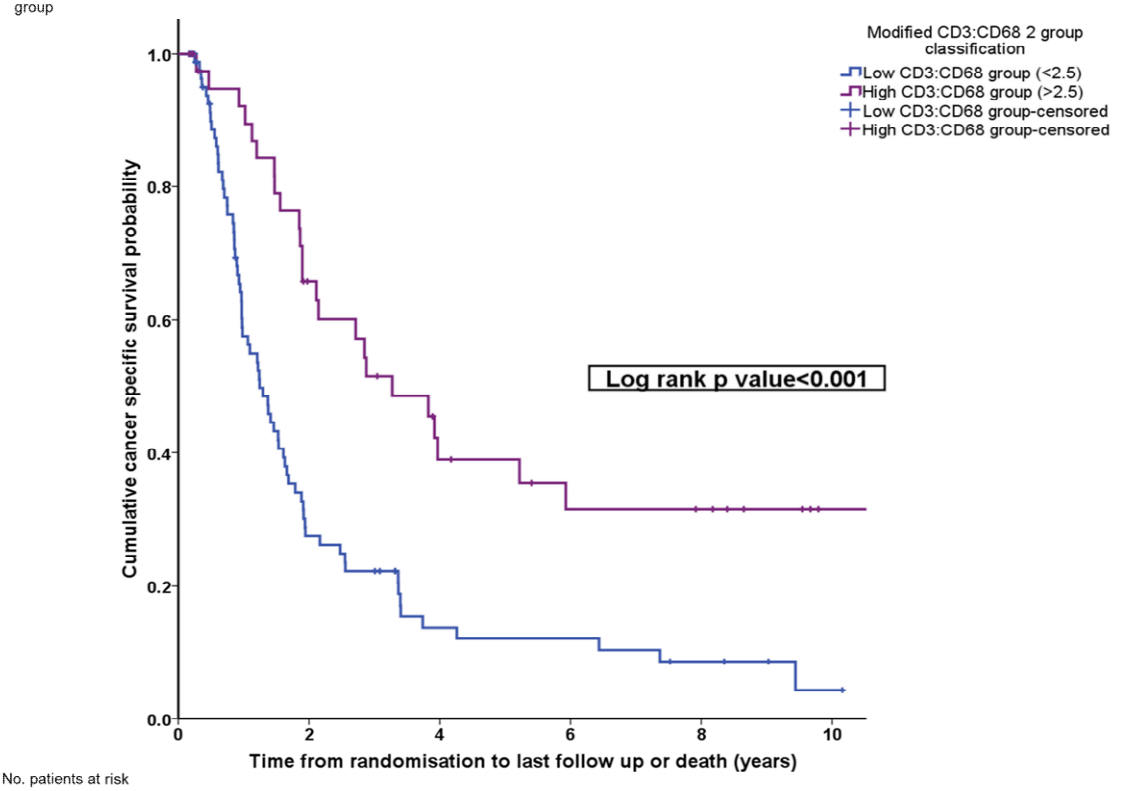
Figure 38(a-d): Kaplan Meier survival plots of cancer specific survival stratified by CD3 to CD68 classification systems (a= 5 groups, b= 4 groups c= 3 groups and d= 2 groups).
The four Kaplan Meier survival plots starting with the 5 group classification top and the 2 group classification bottom show all 4 systems have significant differences in survival. Patients with a high ratio of T cells to macrophages have a statistically significant improved cancer specific survival.

A modification 2 group system was tested by combining the low and mid group patients (ratio <2.5) which could be used against the high group (ratio>2.5). Kaplan Meier survival plots for overall and cancer specific survival (Figure 39) show a significant difference between these two modified groups ($p<0.001$ for both overall and cancer specific survival). There is a significant improved survival in the high CD3:CD68 patients with 11 patients still alive at the end of follow up (25%) compared to 8 from the low CD3:CD68 group (8.9%). Cancer related deaths are also reduced in the high CD3:CD68 group (56.8%) compared to the low CD3:CD68 group (76.7%).



No. patients at risk

Low CD3<CD68 group	90	23	10	8	4	1
High CD3>CD68 group	44	24	13	8	7	1



No. patients at risk

Low CD3<CD68 group	88	21	8	7	4	1
CD3>CD68 group	42	23	12	8	7	1

Figure 39: Kaplan Meier survival plots for overall and cancer specific survival in the chemotherapy and surgery treatment group stratified by a modified 2 group CD3:CD68 ratio system

However, as there seemed to be good prognostic value through most variations of the 3 and 2 group classifications a simple ratio cut-off of 2 was tested. This would essentially divide patient into those who had double the amount of CD3 to CD68 against those who had less (named the simplified 2 group classification, Figure 40). This was hypothesised could possibly be calculated even without access to automated scoring technology. The Kaplan Meier plot for this simplified 2 group classification not only shows a clear survival difference between these two groups, but this cut off like the previous classification systems shows a statistical difference on log rank comparisons for both overall and cancer specific survival ($p=0.001$ and 0.002 respectively). Therefore for simplicity, this simplified 2 tier classification would be the favoured method of determining T cell to macrophage ratios where automated scoring access may not be available (Figure 40).

Overall survival rates between the two groups show that at the end of follow up from randomisation, patients in the low CD3:CD68 ratio group (ratio<2) only 6.7% of patients were still alive (5 out of 75 patients) compared to 23.7% of patients in the high CD3:CD68 ratio (ratio>2) group (14 out of 59 patients). This difference is still evident when looking at cancer specific survival, in the low CD3:CD68 group 78.7% of its patients had died related to their disease (n=59) compared 59.3% of patients from the high CD3:CD68 (ratio>2) group who died related to their primary cancer (n=35).

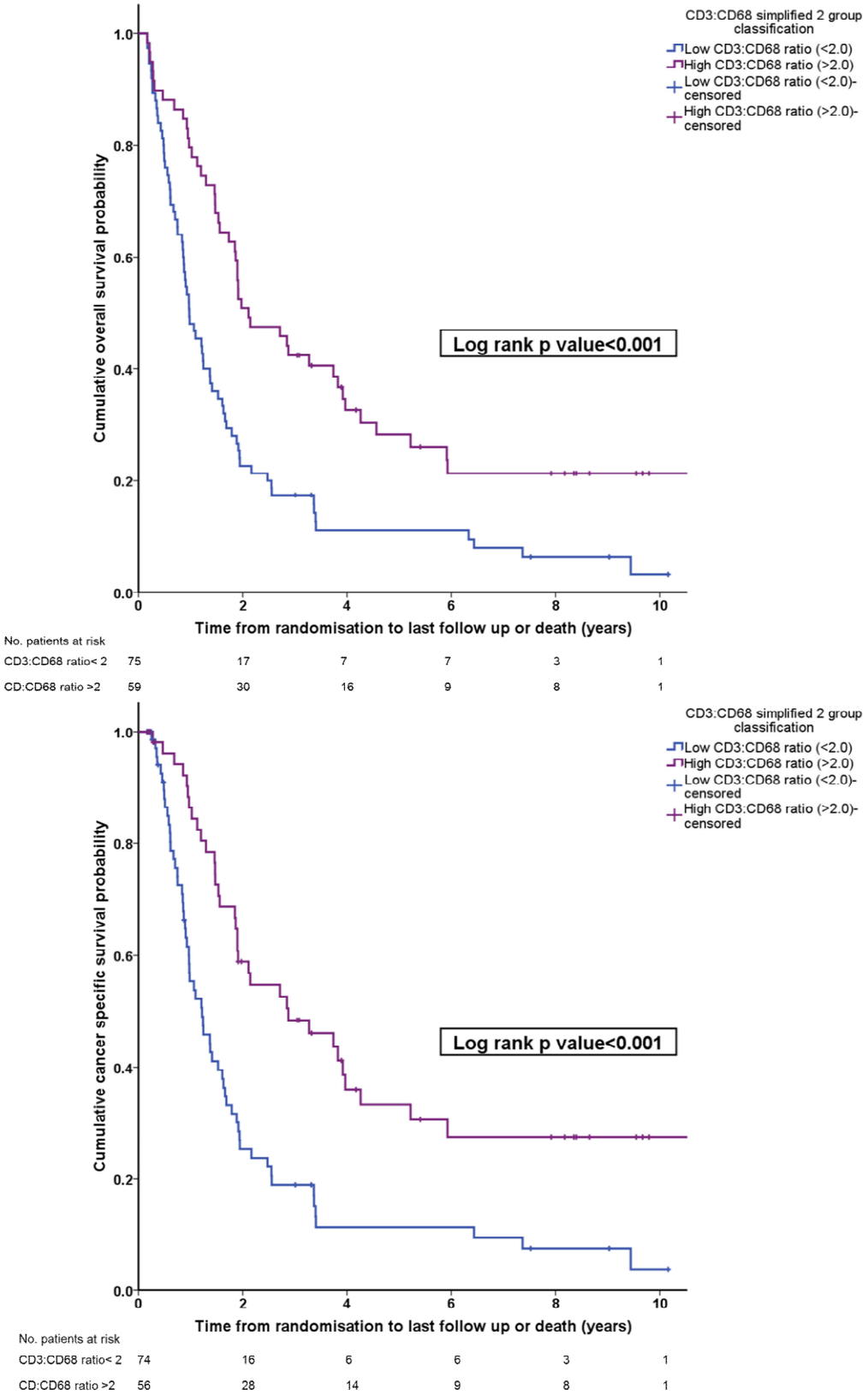


Figure 40: Kaplan Meier survival plots for overall and cancer specific survival stratified using the simplified CD3:CD68 2 group classification.

This simplified 2 group classification is assessed for its survival stratification value in the Kaplan Meier plots above for overall (top) and cancer specific survival (bottom). Cases with greater than 2 times the ratio of CD3 to CD68 have a significantly better survival ($p<0.001$).

The differences in the clinicopathological variables between the simplified 2 group classification (above or below 2) are assessed in Table 48.

Table 48: Relationship between clinicopathological variables and the simplified CD3:CD68 groups using the cut-off ratio of 2.0

	Low CD3:CD68 n=75	High CD3:CD68 n=59	
	Number (%)	Number (%)	P value
Age			
Median	61.6	59.6	0.271
Range	36.4- 83.1	41.6- 76.2	
Gender			
Male	60 (80.0)	48 (81.4)	0.844
Female	15 (20.0)	11 (18.6)	
Histology			
Adenocarcinoma	55 (73.3)	50 (84.7)	0.101
Squamous	18 (24.0)	9 (15.3)	
Other	2 (2.7)	0 (0.0)	
Grade of differentiation			
Poor	35 (46.7)	18 (30.5)	0.132
Moderate	33 (44.0)	35 (59.3)	
Well	6 (8.0)	5 (8.5)	
Unknown	1 (1.3)	1 (1.7)	
ypT category			
T1	2 (2.7)	6 (10.2)	0.182
T2	6 (8.0)	7 (11.9)	
T3	67 (89.3)	44 (74.6)	
T4	0 (0.0)	2 (3.4)	
ypN category			
N0	21 (28.0)	26 (44.1)	0.054
N1	54 (72.0)	33 (55.9)	
Lymphatic vessel invasion			
Yes	29 (38.7)	12 (20.3)	0.218
No	42 (56.0)	41 (69.5)	
Suspicious	4 (5.3)	6 (10.2)	
Vascular invasion			
Yes	7 (9.3)	4 (6.8)	0.053
No	60 (80.0)	54 (91.5)	
Suspicious	8 (10.7)	1 (1.7)	
Mandard TRG			
TRG1	0 (0.0)	0 (0.0)	0.835
TRG2	1 (1.3)	0 (0.0)	
TRG3	8 (10.7)	2 (3.4)	
TRG4	26 (34.7)	27 (45.8)	
TRG5	40 (50.3)	30 (50.8)	

Mann-Whitney non-parametric testing did not identify any statistically significant difference in clinicopathological variables between these two immune cell ratio groups, suggesting that

these immune cells changes are independent of other variables such as patient age or ypT or ypN category.

5.4 Discussion

This work has shown that the amount of infiltration of different immune cells in the tumour microenvironment varies between the two treatment arms of the OE02 trial. In tumours that had undergone neoadjuvant chemotherapy, higher levels of leukocytes in general, as well as specifically T lymphocytes were identified, although not statistically significant. This is in keeping with previous studies in OeC looking at lymphocyte infiltration after neoadjuvant chemotherapy (256) and chemoradiotherapy (248, 249). However the most important prognostic marker established in this work was a ratio score of T lymphocytes to macrophages, which significantly stratified patients into prognostic groups for overall and cancer specific survival. To date this is the first such study to look at this ratio in OeC and follows on from studies in breast (237) and urological malignancies (257). On multivariate cox regression analysis this lymphocyte to macrophage ratio is identified as an independent prognostic marker for overall and cancer specific survival.

The role of the immune system in tumour protection and elimination was first hypothesised over 100 years ago by Peter Ehrlich (444). This hypothesis was followed and supported by subsequent work (445, 446) which highlighted the importance of the immune system's interaction with tumour development. However there were other studies later in the century which raised doubts over any true immune system role in tumour elimination (447-451). This doubt over the role of immune cells in cancer remained until the 1980s and 1990s when studies on immunosuppressed mice showed increased tumour formation occurred in this genetically modified state (223, 452-457). This work in mice specimens was then taken forward by Van Der Bruggen et al in multiple studies which identified the presence of tumour specific antigens in humans which were thought to have possible immune cell target potential (349, 458-460). This discovery lead to a variety of different studies, from various

fields of medicine, all of which were brought together in two large systematic reviews of the literature into the role of immune cells in cancer carried out by Dunn et al (217, 218). These reviews importantly concluded that the immune systems response to cancer cells plays an important role not only in tumour regression but can also influenced tumour progression.

This historical path has led to the modern emphasis on the use of immunotherapy approaches in the treatment of multiple cancer types and a growing interest in research related to immune immunotherapy is ongoing (461-465). Currently two tumours graded identically, using recognised established markers such as TNM staging, can have very different outcomes regardless of getting identical treatment (466). Part of the reason for such discrepancies is patient factors such as existing co-morbidities or age. However these individually patient factors cannot be solely the reason as even in matched cohorts such as the OE02 trial, differences in outcome can still be noted between two tumours of identical TNM staging (84). The impact of different amounts and varying distribution patterns of immune cell infiltration at the tumour microenvironment has been demonstrated in studies of breast (369, 370), lung (371), skin (372), prostate (331) and colorectal cancer (198, 344, 373, 374). In one of Dunn et al reviews they suggested that both the innate and the adaptive immune system participate in host protection from cancerous cells (217, 218). Dunn described the tumour immune system interaction as a two way process involving both immunosurveillance, where the immune cells recognised tumour antigens and immunoediting, where the immune cells could also influence tumour progression (218).

T lymphocytes are strongly indicated as being crucial in tumour control thought to be as a result of tumour cells expressing antigens, enabling them to be targeted by the T cell mediated adaptive immune response (228, 349).T cells were first shown to be associated with cancer prognosis in a study looking at melanomas, which found tumours with a high infiltration of specifically CD8 T cells (cytotoxic cells) had a longer survival than those with low CD8 infiltration (375, 467, 468). This work in melanomas has been followed by

subsequent studies which confirmed that a high infiltration of T cells (generally or Cytotoxic specific T cells) correlated with a good prognosis for various tumours including ovarian (386), lung (226, 227, 229, 469), pancreatic (470, 471) and colorectal cancers (378, 379, 383, 429, 472). A study in 2006 looking at T cells in colorectal cancer suggested that the density of these immune cells in the tumour microenvironment had such importance that their infiltration had superior prognostic value than that of the TNM classification (383). A recent large meta-analysis showed agreement with the findings of this OeC study with regards to prognostic value of high T cell lymphocytes infiltration in the tumour. The meta-analysis included 52 studies which looked specifically at tumour infiltrating lymphocytes in various solid tumours; 10 ovarian, 10 colorectal, 7 lung, 5 hepatocellular, 3 renal and 13 from other cancers sites (230). The results showed an overall survival benefit for patients with a high CD3 T cell infiltration (pooled HR 0.58, 95% CI of 0.43–0.78 for death) and/or high levels of CD8 infiltration on pooling of data (HR 0.71, 95% CI of 0.62–0.82). However no study included in this meta-analysis involved OeC patient. From collective literature it is clear that the infiltration of lymphocytes indicates a form of ‘anti-tumour’ immune response (230), with high levels of T cells likely to correlate with a greater recognition and response from the host immune system to the tumour (473).

The results of this study support this previous literature; however tumour infiltration of T lymphocytes prognostic value is only identified in the neoadjuvant chemotherapy and surgery treatment group. In this group of patients’ tumours with high CD3 staining showed a statistically significant overall survival benefit compared to those with low level staining. Using Kaplan Meier plots initially beginning with even sized groups, it was established that using 6% cut-off produced the optimum cut-off for overall survival stratification. This is the first study which has assessed the prognostic influence of tumour infiltrating lymphocytes in OeC using a homogenous cohort of neoadjuvant chemotherapy followed by surgery patients (as the two OE02 treatment groups were analysed separately). Previous studies in OeC

have shown in patients who have undergone surgery alone that T lymphocyte infiltration was associated with a significant improvement in survival (250-252, 254, 255). The reason T cell infiltration was not found to have prognostic value in surgery alone tumours in this study does not appear to be due to the quantity of lymphocytes between treatment groups as there was no statistically significant difference between treatment groups. Other studies have also not found significant differences in T cell amounts in tumours who have or have not undergone any neoadjuvant treatment (474, 475). Tsuchikawa et al compared tumour microenvironments in OeC patients who had or had not undergone neoadjuvant chemotherapy and found no significant difference in CD8 cells levels in what they defined the tumour nest (256). However no survival analysis was performed in this study to compare with. Zingg et al studied the OeC tumour microenvironments in patients who did (18.5%) or did not receive neoadjuvant chemoradiotherapy (249) and also showed no difference in T lymphocyte infiltration between tumours from the two treatment groups. In agreement to this study although CD3 infiltration levels were significantly associated with overall survival it was not shown to be an independent prognostic marker. So it's possible that the lymphocytes seen at the OeC microenvironment in surgery alone although present in similar quantities, maybe lymphocytes of different qualities to the ones present in the chemotherapy followed by surgery tumour microenvironment. Previous studies have suggested T cells can remain in an "inactive" state and it's only once tumour cells have been affected by the destructive actions of chemotherapy that these lymphocytes become activated (476). However comparing existing studies is difficult due to the heterogeneity between all the existing studies which was commented on in the recent meta-analysis study which did not include any OeC study (230). Previous OeC studies have also suffered from such heterogeneity in study designs with all examining tumour infiltrating lymphocytes have used selected histological cohorts often only squamous cell carcinomas (248, 251-253, 255, 256). Most have examined the whole tumour using various defined areas for scoring (nest vs periphery vs stroma), with some identifying 2 scoring regions (248-251) and others scoring at 5

different areas then taking a median (252, 255, 256). None of these previous studies used automatic scoring technique which was used in this study to reduce any observer subjectivity.

Only one previous study has used similar automated scoring technique as the one used in this study (254). In concordance with this study their cohort of 118 adenocarcinomas who underwent surgery alone failed to show cytotoxic T cell infiltration (CD8) had any prognostic influence on overall survival in their cohort. Their results did indicate that CD3 infiltration did have prognostic value in surgery alone patients which is in disagreement with this thesis. However the scoring technique used was very different to the one used in this study in that 10 randomly selected areas on the TMA were analysed at 400x magnification to produce an index of T cells. This index was taken as the number of “immuno-positive” nuclei visualised divided by the total number of cells. The use of an image analysis system (TMAi) like in this study, has given rapid and reproducible calculations of the individual immune cell infiltration by calculated the percentage of positive pixels (POPP) staining per core. Using manual scoring as a technique involves an often difficult process of reviewing 0.6mm tissue cores under light microscopy, with each core being reviewed individually under high magnification and a scoring system used to attempt to quantify the amount of immune cells staining. This scoring can often involve an estimation of the percentage of staining or actual counting of immune cells which in the situation of having multiple cells can be a difficult task both of which can lead to a large degree of observer variability. The selection of perceived high density scoring areas can also introduce further subjectivity and selection bias despite observers being kept blind to the treatment per case. This study also has the advantage of including material from a randomised control trial carried out at multiple centres.

Our work has shown significantly higher Foxp3 in surgery alone treatment group but no prognostic value in survival analysis. This mirrors the existing evidence in OeC studies involving neoadjuvant treatment followed by surgery (248, 249, 256) and surgery alone

(252). As well as this, pooled data from a recent meta-analysis (described previously) looking at tumour infiltrating lymphocytes in various cancers showed Treg cells infiltration at the tumour microenvironment had no statistically significant impact on overall or cancer specific survival (230). Although Treg cells clearly have an important role in the human immune system, there appears to be no prognostic impact of the amount of these cells infiltrating oesophageal cancer either after neoadjuvant chemotherapy followed by surgery or surgery alone.

Tumour infiltration by macrophages has been demonstrated to have a poor prognostic impact in various tumours (225, 240, 405, 477-479) including oesophageal cancer (398, 431-434). They are associated with tumour progression through angiogenesis promotion and lymphatic invasion (224, 239). However in this study macrophages have not been demonstrated to have any prognostic value in either OE02 treatment group. Previous literature involving OeC and tumour infiltrating macrophages has tended to involve squamous cell carcinomas after surgery alone, carried out on Asian populations (398, 432, 434). With the exception of one, all the previous OeC studies in tumour infiltrating macrophages have attempted to estimate levels using manual counting techniques (398, 432-434). This is in contrast to this study which has used quantitative automated scoring of the amount of immunohistochemistry staining using an established computer program used before in similar work (438, 439). Counting areas varied between previous studies with one study scoring at a single high density area (432), two studies scoring at 3 areas (433, 434) and one study scoring at 5 areas (398). This and the different magnifications used results in macrophage scores not being able to be compared across studies. Sugimura et al did include patients who had neoadjuvant chemotherapy followed by surgery in their study (433). They identified no difference in macrophage infiltration between patients who had undergone neoadjuvant chemotherapy and those who had surgery alone (433). They also did not show the amount of CD68 staining to be a significant prognostic marker in

concordance with this work set out in this chapter. In this study there was a significant difference in macrophage infiltration between the treatment groups (represented by higher POPP of CD68 in the surgery alone case). Higher macrophage levels were associated with poorly differentiated tumours that were more likely to have positive lymph nodes in the surgery alone group. However these associations were not seen in the chemotherapy and surgery group. Whether these higher levels in surgery alone are as a result of chemotherapy reducing “pro-tumour” macrophage numbers is unclear, but a recent study suggested macrophage infiltration is higher in patients who show no evidence of responding to chemotherapy leading them to state that macrophages had a role in chemoresistance (433). The results of studies looking at macrophages in colorectal cancers have gone against the tide in terms of literature into the prognostic effect, with multiple studies showing macrophage presence being a good prognostic marker. This anti-tumour effect shown in these colorectal studies has been suggested as being due to a different phenotypical make up of their tumour invading macrophages (236, 384, 480). Whether there is also a different make up in the phenotype of macrophages found in OeC with and without neoadjuvant therapy has not been tested in this work. Sugimura concluded that tumour infiltrating macrophages where associated with poor prognosis in OeC not because of the results from CD68 staining but from CD163 whose levels were significant associated with overall survival. In recent studies the immunohistochemistry stains CD163 or CD204 have been utilised to try to specifically stain for M2 macrophages which are thought to be the subset of macrophages responsible for tumour progression (224). Both of these stains have been suggested as being more specific at identifying M2 macrophages than using CD68 as was used in this study (234, 236, 434, 481). However, the ability of both stains to selectively stain M2 macrophages is still under debate. There is evidence that CD163 is also expressed by dendritic cells (482) and that CD204 immunohistochemistry stains the same cells as CD68, a pan-macrophage stain (405). With further work a consideration to include CD163 as subset stain for macrophages will be considered.

This current work has created an immune cell ratio using T cell and macrophage infiltration calculations at the actual tumour microenvironment as opposed to using peripheral blood levels as a surrogate marker. From the results of the survival analysis it's clear that it's not the individual infiltration levels of 'anti' tumour T cells and 'pro' tumour macrophages but instead the ratio of T lymphocytes to macrophages (CD3:CD68) which holds the significant prognostic value. There have been various studies which have looked at the lymphocyte to macrophage ratio in blood tests of haematological cancer patients and demonstrated this has prognostic value (483-485). Following this work in haematological malignancies other work examining the importance of blood levels of these two immune cells in different solid tumours such as pancreatic cancer, soft tissue sarcomas, gastric cancer and colorectal cancers, have all found prognostic value in the ratio from pre-treatment blood tests (486-489). However, only three previous studies have examined the infiltration ratio of lymphocytes to macrophages at the tumour itself, two in breast cancer (237, 490) and the other in urological carcinomas (257).

Using this ratio at the tumour microenvironment has the advantage of being able to be used post operatively and even potentially used in endoscopic biopsy material during diagnostic staging or even through neoadjuvant treatment. Using established immunohistochemistry techniques to stain tissue microarrays and then an automatic image analysis system was used to provide a quantitative staining score for each core. The ratio is then easily calculated by dividing the T cell score and the macrophage score which removes any subjectivity to the scoring technique. The results show that a high T cell to macrophage ratio has strong prognostic value in oesophageal cancer. Using cox regression analysis including other recognised prognostic markers such as age, T category, N category and blood vessel invasion, the T cell to macrophage ratio is shown to be an independent prognostic marker for both overall and cancer specific survival. This is in agreement with the previously published studies looking at this ratio in breast and urological malignancies (237, 257, 490). Only ypN

category (TNM6) was also shown to be an independent prognostic marker alongside this T cell to macrophage ratio. Furthermore our results have demonstrated a simple cut off can be used by dividing patients into those with double the CD3 to CD68 staining and those with a lower ratio will give clear prognostic. This simple measure could be done automatically as we have done here or even possibly manually in centres where access to automatic scoring systems may not be possible. This is clearly something that would need to be tested in future studies.

A limitation to the study was that there were different cohorts for each stain due to variations core inclusion. This also meant that not all the cores had a matching T lymphocyte and macrophage POPP scores to create an immune cell ratio. This result from the sectioning of the TMA blocks. Once each TMA block was created they were then sectioned at different levels through the block to create the slices for mounting on to slides. This meant that the quality of each slide varied slightly and some cores as a result were sectioned at different levels. So where a core may have been acceptable for one stain it may not have been for other stains which resulted in the drop out described in Table 30. This tissue loss during sectioning transfer and staining is a recognised limitation of using TMA blocks with a reported dropout rate ranging from 10-30% (491). In this study across the stains the median dropout rate was 20.9% in keeping with previous work. However despite these case drop outs the study still involved a median of 337 cores per stain which is still more than previous studies looking at tumour infiltrating lymphocytes in oesophageal cancer (248, 249). There were also 134 cases assessed with a CD3 to CD68 ratio which is similar size to the two previous similar ratio studies published (237, 257). The use of TMA staining for the assessment multiple cases has been criticised for the small size of the cores with some arguing that such a small sample of a tumour tissue cannot reflect the concentration of the measured cell throughout the whole area (492, 493). Although assessing immune cell concentrations in the tumour using whole sections would have reduced this doubt, it would

have been more time and resource consuming especially with over 500 cases to be assessed. There is also evidence showing that whole section staining can lead to over interpretation and focal staining patterns (494). The technique of using TMAs has been validated in large studies in tumours of the breast (494), lung (495), stomach (438) and colon (496) as well as being used previously in similar OeC studies (254, 431).

Another limitation was in having to manually score the CD20 (B cells) and CD66b (neutrophils) stained cores due to the computer program not being able to distinguish clearly between true and negative staining. Previous studies using this stain have used manual counting techniques to score these stains and in future maybe the first line technique when trying to assess for B cell and neutrophil infiltration at the tumour. Manual scoring just identified cases with any evidence of staining no matter how abundant or how few cells present which may have led to both stains being show as having no prognostic survival value on cox regression analysis. Previous studies into neutrophils at the tumour microenvironment have used manual counting of stained neutrophils in each case then identify the median score before dividing their cohorts into high and low groups (246, 417, 421). However there have been previous studies who have also used this method of scoring neutrophils as either present or absent (420). Although in this work which looked at neutrophil infiltration in melanomas the present cases were further divided into abundant cells and minimal cells which may have helped create enough stratification to enable that study to find neutrophils were significantly related to poor prognosis .

There has been recent criticism of the use of CD68 staining to represent macrophages in clinical human studies and it has been suggested that CD68 can be found on non-macrophage cells including cancer cells (497, 498). CD68 is a glycoprotein is that it is a specific marker for tumour associated macrophages in mice, however in humans its expression is more widespread including being present on granulocytes fibroblast and endothelial cells (498). As well as this non-selective concern, there is also growing evidence

about the two opposite tendencies of the two macrophage phenotypes M1 and M2. M1 “classical” macrophages exhibiting anti-tumour character while M2 “alternative” macrophages being the ones shown to have the pro-tumour effects (405, 481). Recent work using selective stains for M2 macrophages through detection of macrophage scavenger receptors via CD163 or CD204 proteins has found high levels are associated with a poor prognosis and hypothesis that it is these M2 cell which are important to assess when considering prognosis (481).

More recent studies in oesophageal cancer infiltrating immune cells have employed techniques where the immune cell levels were assessed both at the periphery of the tumour and more central in immune cell abundant regions which we did not do in this study. The advantage of measuring from different regions is as studies have shown the immune cell infiltration varies in different areas of the tumour (255, 256), so the assessment of a single random biopsy of the tumour may be criticised as not reflective of the immune cell infiltration of the tumour as a whole. However neither of these prior studies carried out any survival analysis to determine if the immune cell levels in either of these locations was of any prognostic value. Ultimately in this study with the random tissue sample taken from the tumour to create the tissue microarray it has been demonstrated to show prognostic value regardless.

6. CONCLUSIONS

This work is an attempt to review the prognostic value of established prognostic markers and identify new prognostic markers for oesophageal cancer treated with neoadjuvant chemotherapy. In the UK since 2002 neoadjuvant chemotherapy followed by potentially curative surgery is the gold standard management for resectable cancers following the results of the OE02 trial. Despite this change in management and continued advances in endoscopic, radiological and surgical fields the prognosis in oesophageal cancer remains poor (9). The work in this thesis used resection material from the OE02 trial to examine some possible new prognostic markers which could have implications in clinical management. This material originates from an international multi-centre randomised control trial that changed the majority of practice in the United Kingdom following its results. Comparison of the original OE02 trial cohort with the cohort used in this thesis shows that despite the loss of cases which were not received by LICAP at the time of this study (508 cases received from the original 802 trial cases), that the cohort used in this work was representative of this OE02 original cohort.

For the tumour cell density work the initial hypothesis were confirmed. It has been demonstrated as a reproducible scoring technique which can be carried out at the luminal surface of resection material on virtual slides. For the calculation of tumour cell density, areas of malignant cells in contact with a luminal surface were identified and scoring grids created using the RandomSpot® (University of Leeds, Leeds, UK) system (199). Using tumour cell density calculations were made in 480 cases from the OE02 trial a significant difference in tumour cell density was identified between patients in the surgery alone group and patients who had undergone chemotherapy before surgery (<0.001). This would indicate that regression from neoadjuvant chemotherapy could be quantified with the TCD scoring. TCD scoring is also shown to correlate with the established Mandard tumour regression

grading system. Through Kaplan Meier survival plots clear survival differences can be seen between TCD groups created by equal division of the cohort by TCD in 5, 4, 3 or 2 groups. However it's by using a combination of the 3 and 2 group cut-offs (modified classification) that the clearest survival benefits are seen between patient groups. Future work could focus on assessing the same TCD at the pre-treatment stage of oesophageal cancer i.e. on biopsy tissue. All cancers of the oesophagus involve biopsies taken which are used for diagnosis and to guide initial management. This biopsy tissue would be suitable for TCD assessment which could then be reproduced at the stage following surgery on the resection material as done in this current work. The comparisons can be made to see whether there are changes in the absence of chemotherapy which could indicate aggressive disease and comparisons to see in the majority of cases where neoadjuvant chemotherapy has been given following the initial biopsy whether the change (expected decrease) in TCD could be more useful prognostic marker than a single isolated TCD calculation. Also this measure of TCD in biopsy tissue could also be done through serial biopsies throughout chemotherapy to monitor response. Such a concept is currently being trialled by using PET imaging to monitor neoadjuvant treatment (320). There is currently also ongoing work looking at whether an automated scoring program can be established (199). The RandomSpot® (University of Leeds, Leeds, UK) program is able to generate spots in an area of interest that is marked out by an observer. The spots are then reviewed individually and scored again by an observer but the next stage would be if the computer program its self could score the cases once the pre-determined area is selected. The program has been designed to recycle clinical data and the future aims would be to develop an algorithm where the programme has enough memory and recognition to identify a tumour cell point automatically.

The area of tumour infiltrating immune cells is one of growing interest in the last 10 years with various solid tumour studies following work in haematological and skin cancers finding that this local immune response is related to prognosis. Again the initial hypotheses were

met in that this study has shown that immune cell infiltration varies between treatment groups. In this current work in surgery alone patients the infiltration levels of immune cells shows no significant prognostic value. In the chemotherapy followed by surgery treatment group this work has shown a statistically significant prognostic impact of high T cell infiltration at the tumour microenvironment. This survival benefit is seen on Kaplan Meier survival plots when cases were divided by their CD3 staining POPP, with stratification into 2 groups using a 6% cut off shown to be the optimal in terms of overall survival. However CD3 staining levels individually were not an independent prognostic marker unless combined with CD68 staining to create a T lymphocyte to macrophage ratio. This is the first such work in oesophageal cancer to report this ratio at the tumour microenvironment following studies in breast (237) and urological cancer (257). As in these two previous studies the lymphocyte to macrophage ratio has been found to be an independent prognostic marker for overall and cancer specific survival for patients who have undergone neoadjuvant chemotherapy followed by surgery. A ratio of cut off of 2 can divide patients into two prognostic groups with a clear survival benefit for those with greater double the CD3 to CD68. This promising finding would need to be reinforced with a further study involving a bigger cohort of patients who had all undergone neoadjuvant chemotherapy. Recent work has shown that the two main macrophage phenotypes M1 and M2 macrophages can be selectively stained using CD163 immunohistochemistry staining to identify M2 macrophages specifically. It's the M2 macrophages that are thought to exhibit the pro-tumour characteristics which enable tumour progression and escape. Further work would focus on trying to establish whether a CD3 to CD163 ratio could give even greater prognostic stratification value. The clinical importance of this work could be in the use of immune cell scoring to help influence post-operative management and guide possible adjuvant therapies, however before this would be considered a much larger trial would need to be conducted. However this initial finding of the independent prognostic value of a lymphocyte to macrophage intratumoural ratio is promising.

Unfortunately as the distance to the CRM was not part of the minimal pathology dataset at the beginning of the OE02 trial, recruitment CRM distances could not be established so the CRM work was carried out on a separate cohort to the other thesis work. However using local hospital and through collaboration with the South Manchester hospital this work was carried on a large cohort of patients (n=232). The analysis has shown no survival benefit in the College of American Pathologist (CAP) definition of a CRM positive margin (at the margin) over that used in the UK by the Royal College of Pathologist (within 1mm of the margin). While both have prognostic value this study has not identified CRM status as an independent prognostic marker in neoadjuvant chemotherapy followed by surgery oesophageal cancer. This work along with other work formed part of a meta-analysis study published recently which showed pooled results matching the work here.

7. APPENDIX

Appendix 1: Rules for grid creation and placement

1. To use circle shaped area instead of square (used in colorectal work) as felt this would enable more luminal surface contact therefore most collaboration with luminal pre-operative biopsies.

2. Use **9mm^2 circles**. Therefore to use 3.3856mm diameter (produces a circle of9.003mm^2)

3. Circle to be positioned on a slide with viable tumour (avoid necrosis/granulation/ulceration) at the surface aiming to have as much contact with the luminal surface as possible

4. If multiple slides with tumour at the surface to use:

a.) Slide with DEEPEST INFILTRATION

If still no distinction to use

b.) Slide with HIGHEST DENSITY tumour

If still no distinction to use

c.) Slide with LARGEST AREA tumour

5. If using 9mm2 circle leads to over 50% of circle containing normal cells to use multiple small circles side by side ***however the total area must remain 9mm2*** (i.e. 2x 4.5mm2 or 3x 3mm2 circles).

6. When the circle contains normal areas beyond the tumour (i.e. with no further tumour beyond the area then instead of scoring these areas as 2 normal stroma, ***they should be scored as 0 indeterminate***.

NB: Areas with high 0 counts should be re-reviewed

7. In cases where there is no evidence of tumour at the luminal surface these cases should be identified for further analysis. For their initial spot counting

a.) The maximal layer depth of tumour should be record

b.) A circle should be made centring on the area where there is the highest density of tumour

c.) the count should be done as normal using 0 for areas of normal cells outside the tumour

8. Spot counting should then be carried out in accordance to the colorectal spot counting protocol version 3 (February 2010),

Circle formation formulas:

1. 9mm circle:

$AREA=\pi r^2$

$Hence\ radius= \sqrt{area} \div \pi$

$Therefore\ for\ a\ 9mm\ area\ circle\ the\ radius=\sqrt{9} \div \pi$

Radius = 1.69mm and diameter (2r) = 3.386

2. 4.5mm circles:

$Radius =\sqrt{4.5} \div \pi$

Radius = 1.197 and diameter used = 2.394

3. 3mm circles:

$Radius = \sqrt{3} \div \pi$

Radius = 0.977mm and diameter (2r) = 1.954

Appendix 2: Data for intra-observer variability analysis for Table 6

	ORIGINAL counts				REPEAT counts				Difference			
Case No.	Tumour (%)	Stroma (%)	Tumour lumen (%)	Other (%)	Tumour (%)	Stroma (%)	Tumour lumen (%)	Other (%)	Tumour (%)	Stroma (%)	Tumour lumen (%)	Other (%)
UGI0724	121 (42.2)	64 (22.3)	17 (5.9)	85 (29.6)	98 (34.1)	114 (39.7)	7 (2.4)	68 (23.7)	-23 (8.1)	+50 (17.4)	-10 (3.5)	-17 (5.9)
UGI0732	98 (34.1)	156 (54.4)	18 (6.3)	15 (5.2)	84 (29.3)	190 (66.2)	8 (2.8)	5 (1.7)	-14 (4.8)	+34 (11.8)	-10 (3.5)	-10 (3.5)
UGI1058	128 (44.6)	145 (50.5)	0 (0)	14 (4.9)	203 (70.7)	55 (19.2)	25 (8.7)	4 (1.4)	+75 (26.1)	-90 (31.3)	+25 (8.7)	-10 -3.5)
UGI1081	50 (17.4)	224 (78)	0 (0)	13 (4.5)	216 (75.3)	52 (18.1)	0 (0)	19 (6.6)	+166 (57.9)	-172 (59.9)	0 (0)	+6 (2.1)
UGI1105	100 (34.8)	163 (56.8)	8 (2.8)	16 (5.6)	196 (68.3)	64 (22.3)	12 (4.2)	15 (5.2)	+96 (33.5)	-99 (54.5)	+4 (1.4)	-1 (0.4)

Appendix 3: Inter-observer TCD score comparison

(4.5.4.2 Case by case TCD agreement)

UGI number	TS score	GH score	Difference in TCD
UGI-1024	41.84%	60.28%	-18.44%
UGI-1107	25.87%	44.04%	-18.17%
UGI-0019	34.39%	35.69%	-1.30%
UGI-0358	28.22%	27.82%	0.41%
UGI-1287	29.33%	28.87%	0.46%
UGI-0128	45.26%	44.24%	1.01%
UGI-0677	34.12%	32.66%	1.46%
UGI-1064	41.32%	39.86%	1.46%
UGI-0062	30.24%	28.37%	1.87%
UGI-0693	47.18%	45.04%	2.15%
UGI-0944	41.37%	39.13%	2.24%
UGI-0913	21.25%	18.25%	3.01%
UGI-0739	38.60%	35.21%	3.39%
UGI-0973	75.52%	72.03%	3.50%
UGI-1162	56.99%	53.50%	3.50%
UGI-0935	16.37%	12.59%	3.78%
UGI-0078	56.10%	51.75%	4.35%
UGI-0297	69.34%	64.69%	4.65%
UGI-2035	40.43%	35.74%	4.69%
UGI-1203	66.33%	61.02%	5.31%
UGI-0066	96.83%	91.37%	5.46%
UGI-0098	37.23%	31.58%	5.66%
UGI-0713	31.71%	25.35%	6.36%
UGI-0084	57.04%	50.52%	6.53%
UGI-0278	56.69%	49.82%	6.87%
UGI-0783	72.89%	65.57%	7.33%
UGI-0215	47.67%	40.29%	7.38%
UGI-0003	85.12%	77.62%	7.50%
UGI-1947	67.02%	59.51%	7.51%
UGI-0275	75.96%	68.34%	7.62%
UGI-0631	60.46%	52.48%	7.98%
UGI-2056	32.09%	24.05%	8.04%
UGI-1946	73.68%	65.61%	8.07%
UGI-0181	72.14%	62.77%	9.38%
UGI-0447	48.78%	38.91%	9.87%
UGI-0149	28.52%	18.50%	10.02%
UGI-0626	47.10%	37.05%	10.05%

UGI number	TS score	GH score	Difference in TCD
UGI-2018	61.32%	51.22%	10.10%
UGI-0068	72.73%	62.06%	10.67%
UGI-0124	57.29%	44.79%	12.50%
UGI-0476	63.54%	50.00%	13.54%
UGI-1144	69.44%	53.13%	16.32%
UGI-0339	71.18%	53.85%	17.33%
UGI-0089	64.62%	47.00%	17.62%
UGI-0570	80.84%	63.07%	17.77%
UGI-0405	70.38%	52.14%	18.24%
UGI-1186	49.83%	29.97%	19.86%
UGI-0480	41.46%	20.70%	20.76%

Appendix 4: Detailed immunohistochemistry protocol

Title: Immunohistochemistry	
Area of application	ST03/OE05/OE02 Clinical Trial Laboratory
Index code/version number	UGI_SOP_005 Version 2
Date of implementation	June 2009
Review interval	12 month review period

List of contents

1. Principle / purpose of procedure

Immunohistochemistry is a technique that allows the localization of proteins in tissue sections by the use of labelled antibodies. The avidin biotin complex (ABC) method is a standard immunohistochemistry method that involves three layers. The first layer is the primary antibody which binds specifically to the antigen in the tissue. The second layer is a biotinylated secondary antibody and the third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by dab to produce a brown product.

2. Personnel or training requirements

1. Qualified biomedical scientist, clinical scientist, research technician

2. New staff to read the sop and go through the procedure step by step with a trained member of staff and to be supervised until fully competent.

3. Specimen requirements

1. 5 micrometre tissue sections on superfrost plus glass slides that have been incubated at 37^{o}c overnight.

4. Equipment

Vortex

Incubation chambers

Dab incubation tray

Coverslips

37^{o}c oven

Nordic houseware microwavable pressure cooker

Plastic slide holder

5. Health and safety

All staff must follow safe practice for dealing with biological material as stated in the Leeds teaching hospitals NHS trust infection control policy including safe handling and disposal of sharps, exposure to blood, spillage and waste.

All human derived products should be treated as potential biohazard.

See departmental assessments for reagents.

6. Reagents

Xylene

Ethanol (100%, 96%, 70%)

Distilled water

Biotin solution (0.04g biotin – sigma b4501 in 200 ml tbs ph 7.4)

Egg block solution (2 egg whites in 200 ml distilled water)

Hydrogen peroxide solution (20 ml h_2o_2 – sigma 216763 in 180 ml distilled water)

0.01 m tbs working solution ph 7.6 (500ml tris stock, 87 g NaCl, adjust to pH 7.6 and top up to 10 l with distilled water)

0.2 m Tris stock solution (24.2 g Tris in 1 l distilled water)

Tbs/triton x solution (500µl triton x in 1.25 l tbs buffer)

Zymed antibody diluent - invitrogen 00-3218

Dako real detection system peroxidase/dab+, rabbit/mouse – dako k5001

Bleach

0.5 % lithium carbonate

Mayer's haematoxylin (1 g haematoxylin – merck 115938, 0.2 g sodium iodate – merck 106525, 50 g aluminium potassium sulphate – merck 101047, 50 g chloral hydrate – merck 102425, 1 g citric acid – merck 100244. Dissolve in 1 l distilled water while heating. Add 10 drops 25% ammonia – merck 105422. Leave for 1 day then ready to use).

Dpx

Biotinylated tyramine stock solution (ez-link- sulfo-nhs-lc-biotin – pierce 21335, tyramine – sigma t2879, dimethylformamide- sigma 200-679-5, 0.1 m Borat buffer pH 8.0 - merck 106303, 0.01 m tbs ph7.6). Dissolve 100 mg ez-link- sulfo-nhs-lc-biotin in 1 ml dimethylformamide. Dissolve 32 mg tyramine in 50 ml 0.1 m borat buffer. Mix the two solutions together and adjust to pH 8.0. Store in the fridge at 4 ^{o}c for 24 hours and then aliquot and store at -20^{o}c.

Biotinylated tyramine working solution (dilute stock tyramine 1:50 with 0.01 m tbs, add 1 µl h_2o_2/ml shortly before use for activation).

Vector stain elite abc kit – vector laboratories pk-6100

Secondary biotinylated antibody - vector laboratories

7. Quality control

When establishing a staining protocol for a new antibody, a dilution curve of the primary antibody needs to be performed to establish the optimal staining pattern.

One positive and one negative multi-tissue control slide is included for each set of test conditions in each staining run. For the negative control the primary antibody is omitted from the procedure. The negative control is included to evaluate non-specific staining and the positive control is indicative of the correct antigen target and staining intensity.

8. Procedure or methodology

8.1 dako real detection system

8.2 tyramine amplification method

8.1

Dewaxing and rehydration

1. Place paraffin slides in slide holder and immerse slides into xylene for 15 minutes (x3).

2. Immerse slides into ethanol for 5 minutes (x3).

3. Immerse slides into distilled water for 10 minutes.

Antigen retrieval

4. Place 200 ml of distilled water into the microwavable pressure cooker.

5. Place slides in slide holder into a small plastic container and fill with the appropriate buffer until slides are completely covered.

6. Place the container in the pressure cooker and lock the lid.

7. Place pressure cooker in the microwave and set at full power.

8. Once the pressure cooker has reached full pressure the yellow pressure gauge will rise. Cook at full pressure for 5 minutes.

9. Release the pressure by carefully removing the red rubber weight.

10. Cool with running water for 10 minutes.

11. Wash in distilled water.

Blocking of endogenous peroxidase

12. Immerse slides into hydrogen peroxide solution for 15 minutes at room temperature.

Blocking of endogenous biotin

13. Immerse slides in egg block solution for 60 minutes at room temperature.

14. Wash slides thoroughly in running water.

15. Wash in distilled water.

16. Immerse slides in biotin solution for 15 minutes at room temperature.

17. Wash slides in tbs/triton x for 5 minutes (x3).

Incubation with primary antibody

18. Dilute primary antibody with zymed and apply 100 μl to each slide. Use zymed on the negative control. Cover slides with a coverslip and incubate flat in a humidified chamber for 1 hour at 37^{o}c or overnight at 4^{o}c.

19. Rinse off coverslips using squirting bottle containing tbs/triton x and wash with tbs/triton x for 5 minutes (x3).

Incubation with secondary antibody

20. Apply 4 drops of dako real link, biotinylated secondary antibody, bottle a. Cover slides with a coverslip and incubate flat in a humidified chamber for 30 minutes at 37^{o}c.

21. Rinse off coverslips using squirting bottle containing tbs/triton x and wash with tbs/triton x for 5 minutes (x3).

Incubation with ABC complex

22. Apply 4 drops of Dako real streptavidin peroxidase, bottle b. Cover slides with a coverslip and incubate flat in a humidified chamber for 30 minutes at 37^{o}c.

23. Rinse off coverslips using squirting bottle containing TBS/triton x and wash with TBS/triton x for 5 minutes (x3).

Incubation with chromogen

24. Dilute dab+ chromogen, bottle c 1:50 in hrp substrate buffer, bottle d. Apply 100 μl to each slide, cover with a coverslip and incubate flat on the dab tray. Watch the reaction developing under the wet microscope to establish optimal incubation time; should be less than 15 minutes.

25. Rinse off coverslips into bleach using squirting bottle containing TBS/triton x and wash with TBS/triton x for 5 minutes (x3).

NB: dab should be discarded into bleach and left overnight before discarded down the sink.

Counterstain

26. Immerse slides in Mayer's haematoxylin for 30 seconds (Mayer's haemtoxylin needs to be filtered daily before use).

Wash in running tap water until water runs clear.

Immerse slides in 0.5% lithium carbonate for 1 minute.

Check slides under the microscope and if not blue enough put the slides back into haematoxylin for 1 minute and repeat the process until satisfied.

Dehydration and mounting

Immerse slides into ethanol for 5 minutes (x3).

Immerse slides into xylene for 15 minutes (x3).

Mount slides using dip straight xylene.

8.2 alternative tyramine amplification protocol

Steps 1-19 as above

Incubation with secondary antibody

20. Dilute vector secondary biotinylated antibody 1:200 with TBS and apply 100 μl to each slide. The secondary antibody has to be single-species and appropriate for the primary antibody. Cover slides with a coverslip and incubate flat in a humidified chamber for 30 minutes at 37°c.

21. Rinse off coverslips using squirting bottle containing TBS/triton x and wash with TBS/triton x for 5 minutes (x3).

First incubation with ABC complex

22. Dilute vectastain elite ABC kit 1:50 in TBS and apply 100 μl to each slide. Make this ABC complex solution 30 minutes before use. Prepare double the volume needed as the slides are incubated for a second time with the ABC complex. Apply 100μl to each slide. Cover slides with a coverslip and incubate flat in a humidified chamber for 30 minutes at 37°c. 23. Rinse off coverslips using squirting bottle containing TBS/triton x and wash with TBS/triton x for 5 minutes (x3).

Incubation with tyramine solution

24. Defrost the appropriate amount of tyramine stock and dilute 1:50 in 10mm tbs. Just before use add 1 μl h_2o_2/ml for activation. Apply 100 μl to each slide, cover with a coverslip and incubate flat in a humidified chamber for 15 minutes at 37°c.

25. Rinse off coverslips using squirting bottle containing TBS/triton x and wash with TBS/triton x for 5 minutes (x3).

Second incubation with ABC complex

26. Use the ABC complex prepared earlier. Apply 100 μl to each slide, cover with a coverslip and incubate flat in a humidified chamber for 30 minutes at 37°c.

27. Rinse off coverslips using squirting bottle containing TBS/triton x and wash with TBS/triton x for 5 minutes (x3).

Incubation with chromogen

As above

Counterstain

As above

Dehydration and mounting

As above

Appendix 5: Tumour Micro-Arrays Maps

There were in total 9 unique tissue micro-array (TMA) maps each containing a mix of tumour, normal and control cores from cases in the OE02 trial.

- AD_1A
- SQ_1B
- Treated 1A
- Treated 2A
- Treated 3A
- Untreated 1A
- Untreated 2A
- Untreated 3A
- Untreated 4A

Core map abbreviations

- Tu or T- labelled a tumour
- N or No= Labelled a normal core
- W= labelled a control core used in the periphery of all maps
- Blank or gap= labelled a built in space designed to orientate each map

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
1	W	Tu-UGI-5	Tu-UGI-5	Tu-UGI-5	No-UGI-5	No-UGI-5	No-UGI-5	C	Tu-UGI-6	Tu-UGI-6	Tu-UGI-6	No-UGI-6	No-UGI-6	No-UGI-6	W
2	W	Tu-UGI-7	Tu-UGI-7	Tu-UGI-7	No-UGI-7	No-UGI-7	No-UGI-7	C	Tu-UGI-8	Tu-UGI-8	Tu-UGI-8	No-UGI-8	No-UGI-8	No-UGI-8	W
3	W	Tu-UGI-36	Tu-UGI-36	Tu-UGI-36	No-UGI-36	No-UGI-36	No-UGI-36	C	GAP	GAP	GAP	GAP	GAP	GAP	W
4	W	Tu-UGI-42	Tu-UGI-42	Tu-UGI-42	No-UGI-42	No-UGI-42	No-UGI-42	C	Tu-UGI-46	Tu-UGI-46	Tu-UGI-46	No-UGI-46	No-UGI-46	No-UGI-46	W
5	W	C	C	C	C	C	C	C	C	C	C	C	C	C	W
6	W	Tu-UGI-74	Tu-UGI-74	Tu-UGI-74	No-UGI-74	No-UGI-74	No-UGI-74	C	Tu-UGI-77	Tu-UGI-77	Tu-UGI-77	No-UGI-77	No-UGI-77	No-UGI-77	W
7	W	Tu-UGI-89	Tu-UGI-89	Tu-UGI-89	No-UGI-89	No-UGI-89	No-UGI-89	C	Tu-UGI-90	Tu-UGI-90	Tu-UGI-90	No-UGI-90	No-UGI-90	No-UGI-90	W
8	W	Tu-UGI-91	Tu-UGI-91	Tu-UGI-91	No-UGI-91	No-UGI-91	No-UGI-91	W	Tu-UGI-92	Tu-UGI-92	Tu-UGI-92	No-UGI-92	No-UGI-92	No-UGI-92	W
9	W	Tu-UGI-107	Tu-UGI-107	Tu-UGI-107	No-UGI-107	No-UGI-107	No-UGI-107	W	Tu-UGI-147	Tu-UGI-147	Tu-UGI-147	No-UGI-147	No-UGI-147	No-UGI-147	W
10	W	Tu-UGI-149	Tu-UGI-149	Tu-UGI-149	No-UGI-149	No-UGI-149	No-UGI-149	W	Tu-UGI-152	Tu-UGI-152	Tu-UGI-152	No-UGI-152	No-UGI-152	No-UGI-152	W
11	W	Tu-UGI-160	Tu-UGI-160	Tu-UGI-160	No-UGI-160	No-UGI-160	No-UGI-160	W	Tu-UGI-165	Tu-UGI-165	Tu-UGI-165	No-UGI-165	No-UGI-165	No-UGI-165	W
12	W	Tu-UGI-192	Tu-UGI-192	Tu-UGI-192	No-UGI-192	No-UGI-192	No-UGI-192	W	Tu-UGI-194	Tu-UGI-194	Tu-UGI-194	No-UGI-194	No-UGI-194	No-UGI-194	W
13	W	blank	blank	blank	blank	blank	blank	W	Tu-UGI-197	Tu-UGI-197	Tu-UGI-197	No-UGI-197	No-UGI-197	No-UGI-197	W
14	W	Tu-UGI-267	Tu-UGI-267	Tu-UGI-267	No-UGI-267	No-UGI-267	No-UGI-267	W	Tu-UGI-200	Tu-UGI-200	Tu-UGI-200	No-UGI-200	No-UGI-200	No-UGI-200	W
15	W	Tu-UGI-268	Tu-UGI-268	Tu-UGI-268	No-UGI-268	No-UGI-268	No-UGI-268	W	Tu-UGI-269	Tu-UGI-269	Tu-UGI-269	No-UGI-269	No-UGI-269	No-UGI-269	W
16	W	Tu-UGI-274	Tu-UGI-274	Tu-UGI-274	No-UGI-274	No-UGI-274	No-UGI-274	W	Tu-UGI-275	Tu-UGI-275	Tu-UGI-275	No-UGI-275	No-UGI-275	No-UGI-275	W
17	W	Tu-UGI-279	Tu-UGI-279	Tu-UGI-279	No-UGI-279	No-UGI-279	No-UGI-279	W	blank	blank	blank	blank	blank	blank	W
18	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
1	W	Tu-UGI-16	Tu-UGI-16	Tu-UGI-16	No-UGI-16	No-UGI-16	No-UGI-16	C	Tu-UGI-21	Tu-UGI-21	Tu-UGI-21	No-UGI-21	No-UGI-21	No-UGI-21	W
2	W	Tu-UGI-28	Tu-UGI-28	Tu-UGI-28	No-UGI-28	No-UGI-28	No-UGI-28	C	Tu-UGI-44	Tu-UGI-44	Tu-UGI-44	No-UGI-44	No-UGI-44	No-UGI-44	W
3	W	Tu-UGI-61	Tu-UGI-61	Tu-UGI-61	No-UGI-61	No-UGI-61	No-UGI-61	C	Tu-UGI-62	Tu-UGI-62	Tu-UGI-62	No-UGI-62	No-UGI-62	No-UGI-62	W
4	W	Tu-UGI-80	Tu-UGI-80	Tu-UGI-80	No-UGI-80	No-UGI-80	No-UGI-80	C	Tu-UGI-81	Tu-UGI-81	Tu-UGI-81	No-UGI-81	No-UGI-81	No-UGI-81	W
5	W	C	C	C	C	C	C	C	C	C	C	C	C	C	W
6	W	Tu-UGI-98	Tu-UGI-98	Tu-UGI-98	No-UGI-98	No-UGI-98	No-UGI-98	C	Tu-UGI-122	Tu-UGI-122	Tu-UGI-122	No-UGI-122	No-UGI-122	No-UGI-122	W
7	W	Tu-UGI-134	Tu-UGI-134	Tu-UGI-134	No-UGI-134	No-UGI-134	No-UGI-134	C	Tu-UGI-140	Tu-UGI-140	Tu-UGI-140	No-UGI-140	No-UGI-140	No-UGI-140	W
8	W	Tu-UGI-142	Tu-UGI-142	Tu-UGI-142	No-UGI-142	No-UGI-142	No-UGI-142	W	Tu-UGI-158	Tu-UGI-158	Tu-UGI-158	No-UGI-158	No-UGI-158	No-UGI-158	W
9	W	Tu-UGI-169	Tu-UGI-169	Tu-UGI-169	No-UGI-169	No-UGI-169	No-UGI-169	W	Tu-UGI-183	Tu-UGI-183	Tu-UGI-183	No-UGI-183	No-UGI-183	No-UGI-183	W
10	W	Tu-UGI-203	Tu-UGI-203	Tu-UGI-203	No-UGI-203	No-UGI-203	No-UGI-203	W	Tu-UGI-233	Tu-UGI-233	Tu-UGI-233	No-UGI-233	No-UGI-233	No-UGI-233	W
11	W	Tu-UGI-271	Tu-UGI-271	Tu-UGI-271	No-UGI-271	No-UGI-271	No-UGI-271	W	Tu-UGI-272	Tu-UGI-272	Tu-UGI-272	No-UGI-272	No-UGI-272	No-UGI-272	W
12	W	Tu-UGI-273	Tu-UGI-273	Tu-UGI-273	No-UGI-273	No-UGI-273	No-UGI-273	W	Tu-UGI-276	Tu-UGI-276	Tu-UGI-276	No-UGI-276	No-UGI-276	No-UGI-276	W
13	W	Tu-UGI-278	Tu-UGI-278	Tu-UGI-278	No-UGI-278	No-UGI-278	No-UGI-278	W	Tu-UGI-280	Tu-UGI-280	Tu-UGI-280	No-UGI-280	No-UGI-280	No-UGI-280	W
14	W	blank	blank	blank	blank	blank	blank	W	Tu-UGI-296	Tu-UGI-296	Tu-UGI-296	No-UGI-296	No-UGI-296	No-UGI-296	W
15	W	Tu-UGI-297	Tu-UGI-297	Tu-UGI-297	No-UGI-297	No-UGI-297	No-UGI-297	W	Tu-UGI-298	Tu-UGI-298	Tu-UGI-298	No-UGI-298	No-UGI-298	No-UGI-298	W
16	W	Tu-UGI-299	Tu-UGI-299	Tu-UGI-299	No-UGI-299	No-UGI-299	No-UGI-299	W	Tu-UGI-304	Tu-UGI-304	Tu-UGI-304	No-UGI-304	No-UGI-304	No-UGI-304	W
17	W	Tu-UGI-305	Tu-UGI-305	Tu-UGI-305	No-UGI-305	No-UGI-305	No-UGI-305	W	blank	blank	blank	blank	blank	blank	W
18	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
0	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	**w**	0
1	**w**	UGI-2 T	UGI-2 N	gap	UGI-3 T	UGI-3 N	UGI-4 T	UGI-4 N	UGI-19 T	UGI-19 N	UGI-25 T	UGI-25 N	UGI-26 T	UGI-26 N	UGI-31 T	UGI-31 N	UGI-64 T	UGI-64 N	**w**	1
2	**w**	UGI-66 T	UGI-66 N	gap	UGI-72 T	UGI-72 N	UGI-82 T	UGI-82 N	UGI-87 T	UGI-87 N	UGI-88 T	UGI-88 N	UGI-94 T	UGI-94 N	UGI-95 T	UGI-95 N	UGI-97 T	UGI-97 N	**w**	2
3	**w**	UGI-100 T	UGI-100 N	gap	UGI-101 T	UGI-101 N	UGI-112 T	UGI-112 N	UGI-118 T	UGI-118 N	UGI-120 T	UGI-120 N	UGI-124 T	UGI-124 N	UGI-132 T	UGI-132 N	UGI-136 T	UGI-136 N	**w**	3
4	**w**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**w**	4
5	**w**	UGI-156 T	UGI-156 N	gap	UGI-181 T	UGI-181 N	UGI-206 T	UGI-206 N	UGI-215 T	UGI-215 N	UGI-217 T	UGI-217 N	UGI-266 T	UGI-266 N	UGI-270 T	UGI-270 N	UGI-288 T	UGI-288 N	**w**	5
6	**w**	UGI-291 T	UGI-291 N	gap	UGI-294 T	UGI-294 N	UGI-320 T	UGI-320 N	UGI-324 T	UGI-324 N	UGI-333 T	UGI-333 N	UGI-335 T	UGI-335 N	gap	gap	UGI-344 T	UGI-344 N	**w**	6
7	**w**	UGI-346 T	UGI-346 N	gap	UGI-352 T	UGI-352 N	UGI-353 T	UGI-353 N	UGI-361 T	UGI-361 N	UGI-362 T	UGI-362 N	UGI-384 T	UGI-384 N	gap	gap	UGI-403 T	UGI-403 N	**w**	7
8	**w**	UGI-405 T	UGI-405 N	gap	UGI-414 T	UGI-414 N	UGI-427 T	UGI-427 N	UGI-436 T	UGI-436 N	UGI-447 T	UGI-447 N	UGI-452 T	UGI-452 N	gap	gap	UGI-460 T	UGI-460 N	**w**	8
9	**w**	UGI-476 T	UGI-476 N	gap	UGI-554 T	UGI-554 N	UGI-561 T	UGI-561 N	UGI-567 T	UGI-567 N	UGI-568 T	UGI-568 N	UGI-570 T	UGI-570 N	gap	gap	UGI-578 T	UGI-578 N	**w**	9
10	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	10
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Treated 1A TMA core map

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
0	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	0
1	w	UGI-585T	UGI-585N	UGI-589T	UGI-588N	gap	UGI-595T	UGI-595N	UGI-598T	UGI-597N	UGI-601T	UGI-600N	UGI-604T	UGI-605N	UGI-610T	UGI-610N	UGI-626T	UGI-626N	w	1
2	w	UGI-628T	UGI-628N	UGI-631T	UGI-631N	gap	UGI-633T	UGI-633N	UGI-634T	UGI-634N	UGI-635T	UGI-635N	UGI-636T	UGI-636N	UGI-637T	UGI-638N	UGI-669T	UGI-669N	w	2
3	w	UGI-670T	UGI-670N	UGI-671T	UGI-671N	gap	UGI-674T	UGI-674N	UGI-675T	UGI-675N	UGI-679T	UGI-679N	UGI-681T	UGI-681N	UGI-682T	UGI-682N	UGI-683T	UGI-683N	w	3
4	w	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	w	4
5	w	UGI-685T	UGI-685N	UGI-688T	UGI-688N	gap	UGI-690T	UGI-690N	UGI-693T	UGI-693N	UGI-694T	UGI-694N	UGI-695T	UGI-695N	UGI-696T	UGI-696N	UGI-701T	UGI-701N	w	5
6	w	UGI-702T	UGI-702N	UGI-704T	UGI-704N	gap	UGI-708T	UGI-708N	UGI-710T	UGI-710N	UGI-712T	UGI-712N	UGI-714T	UGI-714N	gap	gap	UGI-716T	UGI-716N	w	6
7	w	UGI-717T	UGI-717N	UGI-723T	UGI-723N	gap	UGI-726T	UGI-726N	UGI-728T	UGI-728N	UGI-730T	UGI-730N	UGI-732T	UGI-732N	gap	gap	UGI-734T	UGI-734N	w	7
8	w	UGI-736T	UGI-736N	UGI-737T	UGI-737N	gap	UGI-739T	UGI-739N	UGI-741T	UGI-741N	UGI-743T	UGI-743N	UGI-745T	UGI-745N	gap	gap	UGI-747T	UGI-747N	w	8
9	w	UGI-763T	UGI-763N	UGI-707T	UGI-707N	gap	UGI-768T	UGI-768N	UGI-770T	UGI-770N	UGI-772T	UGI-772N	UGI-773T	UGI-773N	gap	gap	UGI-775T	UGI-775N	w	9
10	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	10
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Treated 2A TMA core map

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
0	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	0
1	w	gap	UGI-1065 T	UGI-1065 N	UGI-1053 T	UGI-1053 N	UGI-1119 T	UGI-1119 N	UGI-1212 T	UGI-1212 N	UGI-0969 T	UGI-0969 N	UGI-0972 T	UGI-0972 N	UGI-0973 T	UGI-0973 N	UGI-1083 T	UGI-1083 N	w	1
2	w	gap	UGI-1084 T	UGI-1084 N	UGI-1041 T	UGI-1041 N	UGI-2004 T	UGI-2004 N	UGI-2005 T	UGI-2005 N	UGI-2007 T	UGI-2007 N	UGI-2012 T	UGI-2012 N	UGI-2018 T	UGI-2018 N	UGI-2021 T	UGI-2021 N	w	2
3	w	gap	UGI-2022 T	UGI-2022 N	UGI-2024 T	UGI-2024 N	UGI-2025 T	UGI-2025 N	UGI-2029 T	UGI-2029 N	UGI-2031 T	UGI-2031 N	UGI-2033 T	UGI-2033 N	UGI-2035 T	UGI-2035 N	UGI-2037 (well diff.) T	UGI-2037 N	w	3
4	w	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	w	4
5	w	gap	UGI-2037 (poorly diff.) T	UGI-2037 N	UGI-2048 T	UGI-2048 N	UGI-2050 T	UGI-2050 N	UGI-2051 T	UGI-2051 N	UGI-2191 T	UGI-2191 N	UGI-0564 T	UGI-0564 N	UGI-0920 T	UGI-0920 no normal - gap	UGI-0962 T	UGI-0962 N	w	5
6	w	gap	UGI-0913 T	UGI-0913 N	UGI-1276 T (LN met)	UGI-1276 no normal - gap	UGI-1043 T (node only)	UGI-1043 no normal - gap	UGI-0916 T	UGI-0916 N	UGI-0930 T	UGI-0930 N	UGI-1060 T	UGI-1060 N	gap	gap	UGI-0566 T	UGI-0566 N	w	6
7	w	gap	UGI-0928 T	UGI-0928 N	UGI-1085 T	UGI-1085 no normal - gap	UGI-0910 T	UGI-910 N	UGI-0927 T	UGI-0927 N	UGI-0402 T	UGI-0402 T	UGI-0402 T	UGI-0402 N	gap	gap	UGI-0562 T	UGI-0562 N	w	7
8	w	gap	UGI-0908 T	UGI-0908 N	UGI-0607 T	UGI-0607 N	UGI-0609 T	UGI-0609 N	UGI-0911 T	UGI-0911 N	UGI-1064 T	UGI-1064 N	UGI-1204 T	UGI-1204 N	gap	gap	UGI-1206 T	UGI-1206 N	w	8
9	w	gap	UGI-1207 T	UGI-1207 N	UGI-1213 T	UGI-1213 N	UGI-1214 T	UGI-1214 N	UGI-0958 T	UGI-0958 N	UGI-1205 T	UGI-1205 N	UGI-1876 T	UGI-1876 N	gap	gap	UGI-1877 T	UGI-1877 N	w	9
10	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	10
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Treated 3A TMA core map

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
0	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	0
1	w	UGI-41 T	UGI-41 N	UGI-63 T	UGI-63 N	UGI-67 T	UGI-67 N	gap	UGI-68 T	UGI-68 N	UGI-70 T	UGI-70 N	UGI-73 T	UGI-73 N	UGI-78 T	UGI-78 N	UGI-79 T	UGI-79 N	w	1
2	w	UGI-84 T	UGI-84 N	UGI-85 T	UGI-85 N	UGI-96 T	UGI-96 N	gap	UGI-99 T	UGI-99 N	UGI-110 T	UGI-110 N	UGI-116 T	UGI-116 N	UGI-128 T	UGI-128 N	UGI-130 T	UGI-130 N	w	2
3	w	UGI-138 T	UGI-138 N	UGI-176 T	UGI-176 N	UGI-189 T	UGI-189 N	gap	UGI-213 T	UGI-213 N	UGI-287 T	UGI-287 N	UGI-293 T	UGI-293 N	UGI-313 T	UGI-313 N	UGI-321 T	UGI-321 N	w	3
4	w	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	w	4
5	w	UGI-337 T	UGI-337 N	UGI-339 T	UGI-339 N	UGI-342 T	UGI-342 N	gap	UGI-345 T	UGI-345 N	UGI-348 T	UGI-348 N	UGI-354 T	UGI-354 N	UGI-355 T	UGI-355 N	UGI-357 T	UGI-357 N	w	5
6	w	UGI-386 T	UGI-386 N	UGI-426 T AD	UGI-426 N	UGI-426 T SQ	UGI-426 N	gap	UGI-410 T	UGI-410 N	UGI-438 T	UGI-438 N	UGI-449 T	UGI-449 N	gap	gap	UGI-456 T	UGI-456 N	w	6
7	w	UGI-457 T	UGI-457 N	UGI-466 T	UGI-466 N	UGI-471 T	UGI-471 N	gap	UGI-473 T	UFI-473 N	UGI-480 T	UGI-480 N	UGI-555 T	UGI-555 N	gap	gap	UGI-556 T	UGI-556 N	w	7
8	w	UGI-557 T	UGI-557 N	UGI-559 T	UGI-559 N	UGI-560 T	UGI-560 N	gap	UGI-573 T	UGI-573 N	UGI-576 T	UGI-576 N	UGI-581 T	UGI-581 N	gap	gap	UGI-584 T	UGI-584 N	w	8
9	w	UGI-845 T	UGI-845 N	UGI-848 T	UGI-848 N	UGI-849 T	UGI-849 N	gap	UGI-853 T	UGI-853 N	UGI-854 T	UGI-854 N	UGI-855 T	UGI-855 N	gap	gap	UGI-906 T	UGI-906 N	w	9
10	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	10
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Untreated 1A TMA core map

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
0	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	0
1	w	UGI-0043T	UGI-0043N	UGI-0407T	UGI-0407N	UGI-0429T	UGI-0429N	UGI-0459T	UGI-0459N	UGI-0909T	UGI-0909N	gap	UGI-0919T	UGI-0919N	UGI-0929T	UGI-0929N	UGI-0932T	UGI-0932N	w	1
2	w	UGI-0940T	UGI-0940N	UGI-0942T	UGI-0942N	UGI-0944T	UGI-0944N	UGI-0946T	UGI-0946N	UGI-0948T	UGI-0948N	gap	UGI-0950T	UGI-0950N	UGI-0954T	UGI-0954N	UGI-0955T	UGI-0955N	w	2
3	w	UGI-0957T	UGI-0957N	UGI-0963T	UGI-0963N	UGI-0970T	UGI-0970N	UGI-1005T	UGI-1005N	UGI-1007T	UGI-1007N	gap	UGI-1011T	UGI-1011N	UGI-1012T	UGI-1012N	UGI-1014T	UGI-1014N	w	3
4	w	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	w	4
5	w	UGI-1016T	UGI-1016N	UGI-1018T	UGI-1018N	UGI-1021T	UGI-1021N	UGI-1023T	UGI-1023N	UGI-1024T	UGI-1024N	gap	UGI-1026T	UGI-1026N	UGI-1037T	UGI-1037N	UGI-1039T	UGI-1039N	w	5
6	w	UGI-1045T	UGI-1045N	UGI-1051T	UGI-1051N	UGI-1058T	UGI-1058N	UGI-1079T	UGI-1079N	UGI-1080T	UGI-1080N	gap	UGI-1081T	UGI-1081N	gap	gap	UGI-1082T	UGI-1082N	w	6
7	w	UGI-1086T	UGI-1086N	UGI-1103T	UGI-1103N	UGI-1107T	UGI-1107N	UGI-1109T	UGI-1109N	UGI-1111T	UGI-1111N	gap	UGI-1116T	UGI-1116N	gap	gap	UGI-1152T	UGI-1152N	w	7
8	w	UGI-1191T	UGI-1191N	UGI-1193T	UGI-1193N	UGI-1195T	UGI-1195N	UGI-1196T	UGI-1196N	UGI-1197T	UGI-1197N	gap	UGI-1198T	UGI-1198N	gap	gap	UGI-1199T	UGI-1199N	w	8
9	w	UGI-1200T	UGI-1200N	UGI-1201T	UGI-1201N	UGI-1202T	UGI-1202N	UGI-1203T	UGI-1203N	UGI-1210T	UGI-1210N	gap	UGI-1211T	UGI-1211N	gap	gap	UGI-1319T	UGI-1319N	w	9
10	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	10
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Untreated 2A TMA core map

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
0	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	0
1	w	gap	UGI-1065 T	UGI-1065 N	UGI-1053 T	UGI-1053 N	UGI-1119 T	UGI-1119 N	UGI-1212 T	UGI-1212 N	UGI-0969 T	UGI-0969 N	UGI-0972 T	UGI-0972 N	UGI-0973 T	UGI-0973 N	UGI-1083 T	UGI-1083 N	w	1
2	w	gap	UGI-1084 T	UGI-1084 N	UGI-1041 T	UGI-1041 N	UGI-2004 T	UGI-2004 N	UGI-2005 T	UGI-2005 N	UGI-2007 T	UGI-2007 N	UGI-2012 T	UGI-2012 N	UGI-2018 T	UGI-2018 N	UGI-2021 T	UGI-2021 N	w	2
3	w	gap	UGI-2022 T	UGI-2022 N	UGI-2024 T	UGI-2024 N	UGI-2025 T	UGI-2025 N	UGI-2029 T	UGI-2029 N	UGI-2031 T	UGI-2031 N	UGI-2033 T	UGI-2033 N	UGI-2035 T	UGI-2035 N	UGI-2037 (well diff.) T	UGI-2037 N	w	3
4	w	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	w	4
5	w	gap	UGI-2037 (poorly diff.) T	UGI-2037 N	UGI-2048 T	UGI-2048 N	UGI-2050 T	UGI-2050 N	UGI-2051 T	UGI-2051 N	UGI-2191 T	UGI-2191 N	UGI-0564 T	UGI-0564 N	UGI-0920 T	UGI-0920 no normal - gap	UGI-0962 T	UGI-0962 N	w	5
6	w	gap	UGI-0913 T	UGI-0913 N	UGI-1276 T (LN met)	UGI-1276 no normal - gap	UGI-1043 T (node only)	UGI-1043 no normal - gap	UGI-0916 T	UGI-0916 N	UGI-0930 T	UGI-0930 N	UGI-1060 T	UGI-1060 N	gap	gap	UGI-0566 T	UGI-0566 N	w	6
7	w	gap	UGI-0928 T	UGI-0928 N	UGI-1085 T	UGI-1085 no normal - gap	UGI-0910 T	UGI-910 N	UGI-0927 T	UGI-0927 N	UGI-0402 T	UGI-0402 T	UGI-0402 T	UGI-0402 N	gap	gap	UGI-0562 T	UGI-0562 N	w	7
8	w	gap	UGI-0908 T	UGI-0908 N	UGI-0607 T	UGI-0607 N	UGI-0609 T	UGI-0609 N	UGI-0911 T	UGI-0911 N	UGI-1064 T	UGI-1064 N	UGI-1204 T	UGI-1204 N	gap	gap	UGI-1206 T	UGI-1206 N	w	8
9	w	gap	UGI-1207 T	UGI-1207 N	UGI-1213 T	UGI-1213 N	UGI-1214 T	UGI-1214 N	UGI-0958 T	UGI-0958 N	UGI-1205 T	UGI-1205 N	UGI-1876 T	UGI-1876 N	gap	gap	UGI-1877 T	UGI-1877 N	w	9
10	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	10
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Untreated 3A TMA core map

	0	1	2	3	4	5	6	7	8	
0	w	w	w	w	w	w	w	w	w	0
1	w	UGI-1120 T	UGI-1120 N	UGI-0960 T	UGI-0960 N	UGI-1287 T	UGI-1287 N	c	w	1
2	w	c	c	c	c	c	c	c	w	2
3	w	UGI-1280 T	UGI-1280 N	UGI-1312 T	UGI-1312 N	UGI-1240 T	UGI-1240 N	c	w	3
4	w	UGI-1308 T	UGI-1308 N	UGI-1879 T	UGI-1879 N	UGI-1881 T	UGI-1881 N	c	w	4
5	w	UGI-1042 T	UGI-1042 N	gap	gap	gap	gap	gap	w	5
6	w	UGI-0174 T	UGI-0174 N	UGI-1131 T	UGI-1131 N	UGI-1139 T	UGI-1139 N	gap	w	6
7	w	w	w	w	w	w	w	w	w	7
	0	1	2	3	4	5	6	7	8	

Untreated 4A TMA core map

Appendix 6: Data safeguards

All patient specimens or material were anonymous.

No patient initials or DOB were included in data.

All data was stored on a university secured and backed up network drive.

No information was moved out of this file and stored in other drives.

Logbook was kept in locked draw on university property to reduce the chances of data being lost.

No patient details where ever documented in loose files or logbook entries.

Appendix 7: Ethical approval document

Ethical approval granted 23rd November 2007 by the South East Research Ethics Committee on behalf of the national research ethics services.

Reference Number 07/H1102/111

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