Does the presence of disseminated tumour cells in bone marrow or circulating tumour cells predict early tumour recurrence in patients with oesophagogastric cancer?

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Abstract

Oesophagogastric cancer is associated with poor long term survival; overall five-year survival is 10-15%. A significant proportion of patients who undergo curative surgery subsequently develop metastatic disease. Evidently, local control of the tumour does not eliminate the risk of haematogenic recurrence. The cancer cell biology of disseminated disease and the mechanisms of its development remain poorly understood. The presence of disseminated tumour cells in blood and bone marrow of patients with breast, colorectal and lung carcinoma are associated with poor prognosis.

The aims of the study were to develop a reliable assay to identify circulating tumour cells (CTCs) in the blood and disseminated tumour cells (DTCs) in the bone marrow and characterize the prevalence, biology and heterogeneity of the cells in patients with oesophagogastric cancer by high resolution imaging flow cytometry and fluorescence activated cell sorting. The objective of the thesis was to evaluate the prognostic significance CTCs and DTCs in oesophagogastric cancer.

Blood samples were taken from patients undergoing curative and palliative treatment for oesophagogastric cancer. Bone marrow from the rib section excised as part of an open oesophagetomy was collected. CTCs and DTCs were isolated from the blood and the bone marrow by red cell lysis and immunomagnetic removal of white blood cells. Enriched cells were incubated with antibodies against epithelial, mesenchymal and predictive biomarkers. CTCs and DTCs were identified based upon their morphology and biomarker expression by high resolution imaging flow cytometry.

CTCs and DTCs were present in all patients undergoing curative and palliative treatment for oesophagogastric cancer. Post curative surgery, patients with 100 or more CTCs in the blood had a significant reduction in relapse free survival ($p=0.012$).

The study highlights the prognostic potential of CTCs in the blood and DTCs in the bone marrow of patients undergoing curative and palliative treatment for oesophagogastric cancer.

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Abbreviations

AF488 – Alexa Fluor 488 (fluorochrome)

AF647 - Alexa Fluor 647 (fluorochrome)

AT – Anaerobic threshold

BMTCS – Bone marrow disseminated tumour cells

CEA – Carcinoembryogenic protein (CEA)

CK – Cytokeratins

C-Met – Tyrosine-protein kinase recptor

CPET – Cardiopulmonary exercise testing

CT – Computer tomography

CTC – Circulating tumour cells

CT-PET – Computer tomography and Postitron emission topography

DAPI – 4,6-diamino-2-phenylindole

DMEM – Dulbecco's modified Eagle medium

DNA – Deoxyribonucleic acid

DTC – Disseminated tumour cell

PBS – phosphate buffered saline

ECX – Epirubicin, Capecitabine, Cisplatin

EDTA – Ethylenediaminetetraaceitic acid

EGFR – Epidermal growth factor receptor

EMP – Epithelial to mesenchymal plasticity

EMR – Endoscopic mucosal resection

EMT – Epithelial to mesenchymal transition

EpCAM – Epithelial cell adhesion molecule

EUS – Endoscopic Ultrasound

FACS – Fluorescent activated cell sorting

FGFR2 – Fibroblast growth factor receptor

FCS – Foetal calf serum

GOJ – Gastro-oesophageal junction

HCX – Trastzumab, Capecitabine, Cisplatin

HER2 – Human epidermal growth factor receptor 2

HGD – High grade dysplasia

LGD – Low grade dysplasia

MIO – Minimally Invasive oesophagectomy

NCCC – Northern Centre for Cancer Care

NICR – Northern Institute of Cancer Research

OGD - oesophgaogastroduodenoscopy

PE – R-Phyco-erythrin

RNA – Ribonucleic acid

RPMI – Rosewell Park Memorial Institute

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

TS – Thymidylate Synthase

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Chapter 1 : Introduction

1.1 Anatomy and Physiology

The oesophagus is a muscular tube that is approximately 25-30cm in length and extends from the pharynx to the stomach. It is divided into three parts: cervical, thoracic and abdominal. Figure 1.1.1 illustrates each segment in relation to the anatomical location, bloody supply, nerve supply and lymphatic drainage.

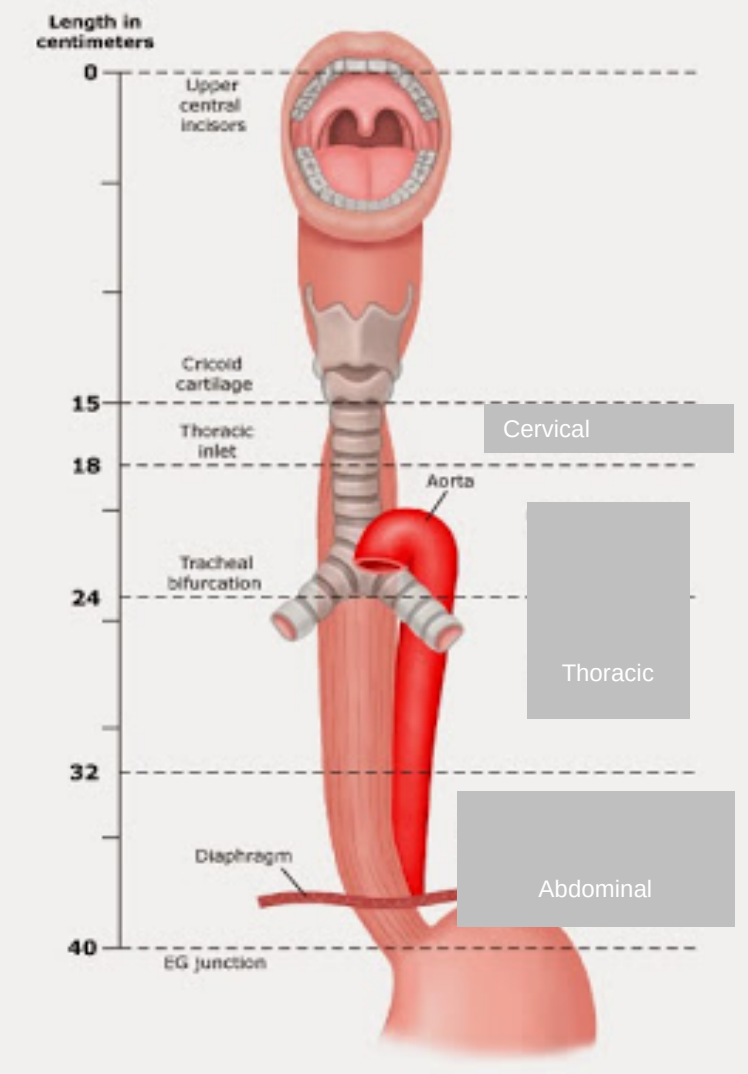


Figure 1.1.1 – Anatomical description of the oesophagus

The oesophagus is a muscular tube which is approximately 25-30 cm in length and extends from the pharynx to the stomach. It is divided into three parts based on its anatomical location and the neurovascular supply: cervical, thoracic and abdominal.

The cervical oesophagus extends from the cricopharynx to the thoracic inlet. The arterial blood supply is from the branches of the inferior thyroid artery, venous drainage of blood is via the inferior thyroid vein and nerve supply from the cervical nerves.

The thoracic oesophagus extends from the thoracic inlet to the diaphragmatic hiatus. The arterial supply in from branches of the descending aorta and venous drainage is to azygous veins.

The abdominal part of the oesophagus extends from the diaphragmatic hiatus to the gastro-oesophageal junction. Arterial blood supply is via the left gastric artery and venous drainage is via the left gastric vein.

Figure taken from https://sites.google.com/a/mtlstudents.net/the-digestive-system/home/the-

The primary function of the oesophagus is the transport of food and liquid from the mouth into the stomach. Muscular contractions along the oesophagus propel the luminal contents from the pharynx to the stomach. The wall of the oesophagus is divided into seven main layers as illustrated in Figure 1.1.2.

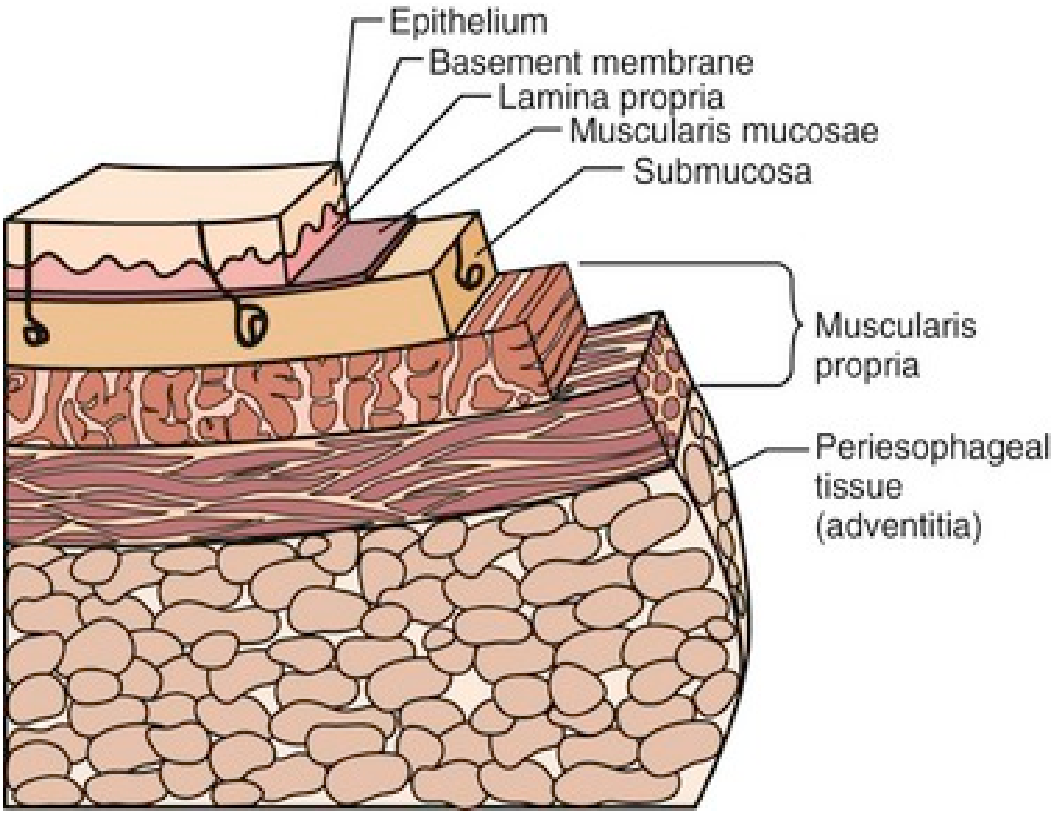


Figure 1.1.2 : Layers of the oesophageal wall

The oesophageal wall is divided into seven layers. The four inner layers consists of the epithelium, basement membrane, lamina propria and muscularis mucosae. Oesophageal cancers confined within these four layers are amenable to endoscopic resection.

Further extension of the cancer into the submucosal layer increases the risk of lymph node metastases and thus is not amenable to endoscopic resection.

The muscularis propria consists of two layers of muscle – longitudinal and circular. The outer layer of the oesophagus consists of the adventitae.

Figure take from: https://thoracickey.com/wp-content/uploads/2016/08/B9781437715606000433_f043-006-9781437715606.jpg

The transition from the stratified squamous epithelium to the columnar epithelium which lines the stomach occurs at the gastro-oesophageal junction.

1.2 Oesophageal cancer

Oesophageal cancer is the 8th most common cancer and is the 6th most common cause of cancer related death worldwide (1). There are two histological subtypes of oesophageal cancer, squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma remains the most common subtype of oesophageal cancer worldwide(2). Oesophageal adenocarcinoma is the most prevalent subtype of oesophageal cancer in the western population. It is associated with poor long term survival; overall 5 year survival is 10- 15% (3, 4). The incidence of oesophageal cancer in the UK has increased over the past two decades as illustrated by Figure 1.2.1 (5). Oesophageal cancer is more common in males than females (Figure 1.2.1 and Figure 1.2.2). The peak incidence of oesophageal cancer is between 65 to 75 (Figure 1.2.1).

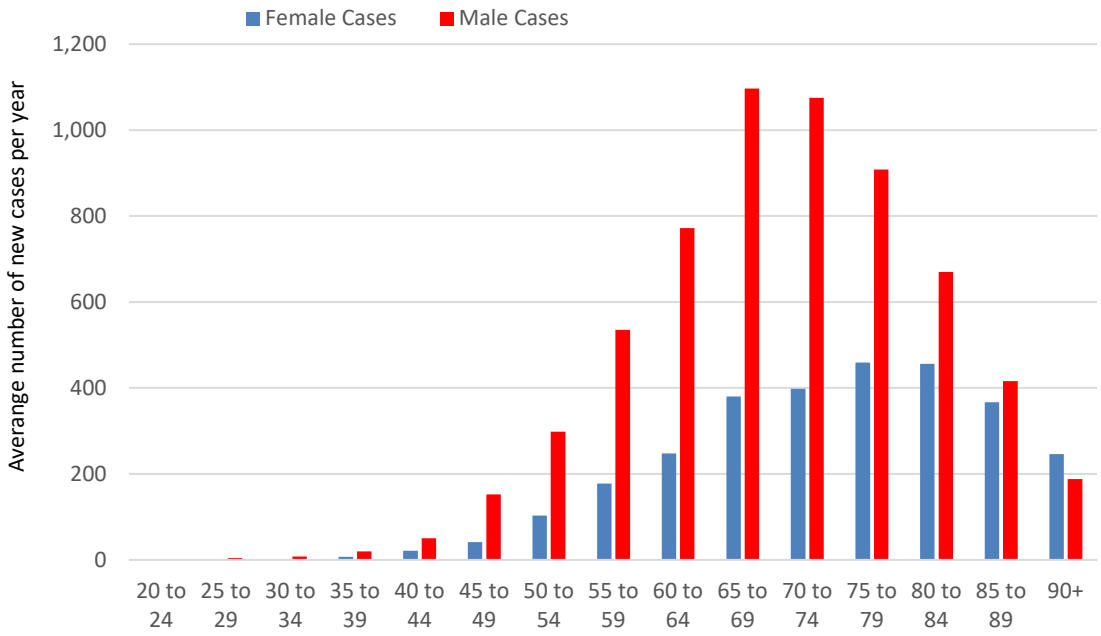


Figure 1.2.1: Age specific incidence rates in the UK per 100 00 cases 2014-2016

The incidence of oesophageal cancer is the highest in the 65-69 age group. The incidence of oesophageal cancer is three times higher in males than in females. Over the last decade the incidence of oesophageal cancer in patients over the age of 80 has been increased. Cancer Research UK https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/oesophageal-cancer/incidence#heading-Two

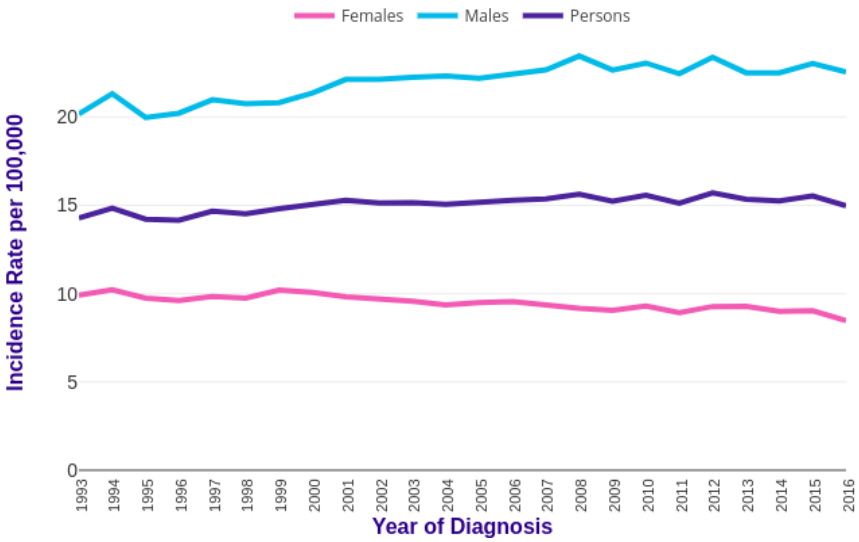


Figure 1.2.2: The incidence of oesophageal cancer in the UK in males and females per 100 000 people between 1993-2016

Incidence of oesophageal cancer in males has steadily increased over the last two decades and is higher than in females. The incidence in females has declined over the last 10 years.

Cancer Research UK https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/oesophageal-cancer/incidence#heading-Two

However over the last decade, improved life expectancy has led to an increase in the number of patients over the age of 80 being diagnosed with oesophageal cancer.

Patients present commonly with difficulty swallowing which arises due to the tumour growth obstructing the lumen of the oesophagus. Unfortunately this occurs at a late stage of cancer development and majority of the patients present with locally advanced disease as illustrated in Figure 1.2.3.

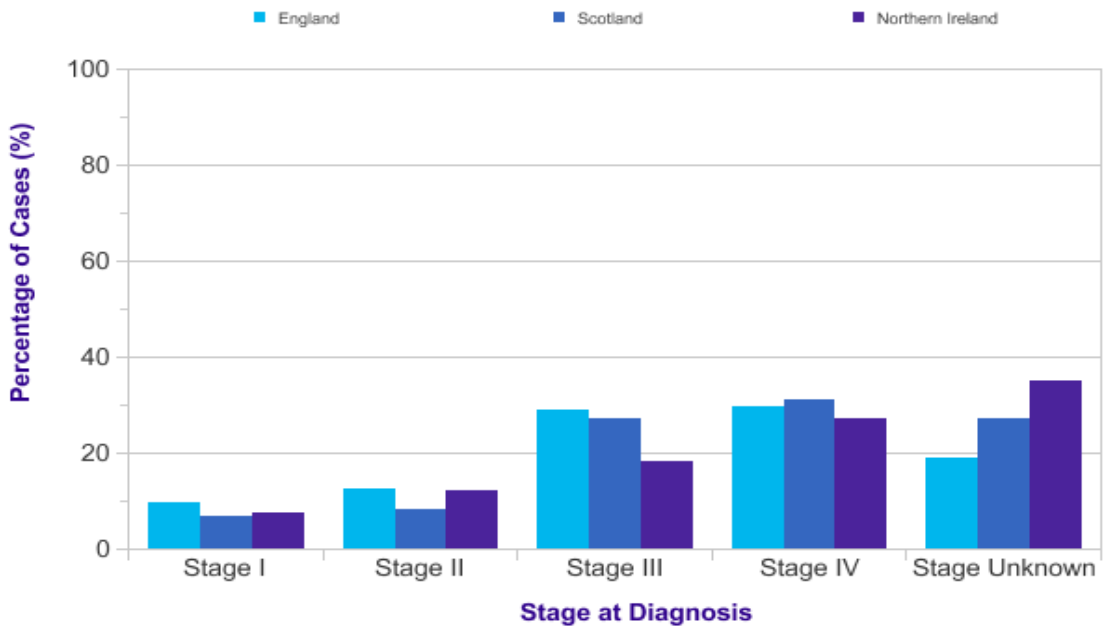


Figure 1.2.3 Proportion of cases diagnosed at each stage of oesophageal cancer at the time of presentation, All Ages, England 2014, Scotland 2014 and Northern Ireland 2010-2014

The majority of patients present with locally advanced cancer, Stage III and IV disease. Approximately 25% of patient present with metastatic disease. The number of patients presenting with early cancer in the UK remains low, less than 10%. Cancer Research UK, https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/oesophageal-cancer/incidence#heading-Two,

1.3 Pathophysiology

Oesophageal adenocarcinoma arises mainly in the distal oesophagus and gastro-oesophageal junction. The primary aetiology factor in the development of oesophageal adenocarcinoma is gastro-oesophageal reflux (6-8). Another contributing factor is obesity. The rise in the incidence of oesophageal adenocarcinoma in the Western population is related to the obesity epidemic that is now prevalent (9). Other risk factor include smoking and alcohol. Gastro-oesophageal reflux results in the exposure of the distal oesophagus to gastric contents which contain gastric acid, gastric enzymes, ingested food and metabolites. The subsequent inflammatory process is thought to contribute to oxidative stress which promotes DNA damage, cellular proliferation and telomere shortening, which can increase the risk of developing clones containing small and

large-scale genomic alterations, eventually leading to widespread chromosomal instability and oesophageal adenocarcinoma(6, 10). One of the steps in the process is the transformation of the stratified squamous epithelium to a columnar epithelium similar to that in the stomach, a process known as metaplasia(6, 7). This pre-malignant condition is called Barrett's metaplasia of the oesophagus and may progress to dysplasia (11). The risk of progression to adenocarcinoma is dependent upon the degree of dysplasia present in the epithelium. The annual risk of progression to adenocarcinoma in non dysplastic Barrett's oesophagus is 0.33% a year and rises to 0.9-1% a year in the presence of high grade dysplasia (11, 12). British Society of Gastroenterologists recommendations are for the endoscopic surveillance of patients with Barrett's metaplasia (11). The frequency of surveillance is dependant upon the length of the segment of Barrett's metaplasia. If the maximum length of Barrett's metaplasia is less than 3 cm, a repeat OGD is required at 3-5 year intervals. If the maximum length of Barrett's metaplasia is greater than 3 cm, a repeat OGD is required at 2 -3 year intervals(11).

However, recent studies have shown that in over 95% of patients who present with oesophageal adenocarcinoma have had no previous diagnosis of Barrett's metaplasia of the oesophagus(12). A meta analysis concluded that the incidence of oesophageal adenocarcinoma in non-dysplastic Barrett's metaplasia was 1 in 300 patients per year and 1 in 500 patients per year in patients with short segment Barrett's metaplasia of the oesophagus(12). The challenge is to identify patients with Barrett's metaplasia of the oesophagus who are at risk of progression to oesophageal adenocarcinoma.

1.4 Management of Oesophageal Cancer

1.4.1 Investigations

Patients undergo a series of endoluminal and radiological investigations to accurately stage the disease. Endoluminal investigations, consist primarily of endoscopy and biopsy with the addition of endoscopic ultrasound (EUS). EUS evaluates the extent of the tumour invasion locally and assesses the nodal involvement of the cancer(13). EUS has been shown to be the most accurate imaging modality in terms of evaluating nodal status (14, 15). However, the accuracy of EUS is dependent upon the operator(13, 15). Computer tomography (CT) of the chest,

abdomen and pelvis evaluates the disease locally and is used in conjunction with positron emission tomography (PET) to identify the presence of distant metastases(13). Patients with cancers at the oesophagogastric junction undergo staging laparoscopy to assess the intra-abdominal component of the tumour and to exclude peritoneal disease. In addition, peritoneal fluid washings taken at staging laparoscopy are analysed for the presence of malignant cells within the peritoneal cavity.

The stage of oesophageal cancer is determined based upon these investigations. Figure 1.4.1 illustrates the TNM staging based on the tumour invasion, nodal status and the presence of metastatic disease (16). Early stage oesophageal cancer is categorised into Tis: high-grade dysplasia (HGD) and T1 which is cancer that invades the lamina propria, muscularis mucosae, or submucosa and is divided into T1a which is cancer that invades the lamina propria or muscularis mucosae and T1b which is cancer that invades the submucosa. Oesophageal cancer staged as T2 denotes invasion the muscularis propria. T3 is cancer that invades the adventitia and T4 is cancer that invades the local structures. T4 cancer can be further classified as T4a which is cancer that invades adjacent structures such as the pleura, pericardium, azygos vein, diaphragm, or peritoneum and T4b which is cancer that invades the major adjacent structures, such as the aorta, vertebral body, or trachea. The N stage denotes the nodal status and is categorized as N0 which is no regional lymph node metastasis, N1 which is regional lymph node metastases involving one to two nodes, N2 which is regional lymph node metastases involving three to six nodes and N3 which is regional lymph node metastases involving seven or more nodes. The M stage is categorized as M0 which is no distant metastasis and M1 which is the presence of distant metastasis.

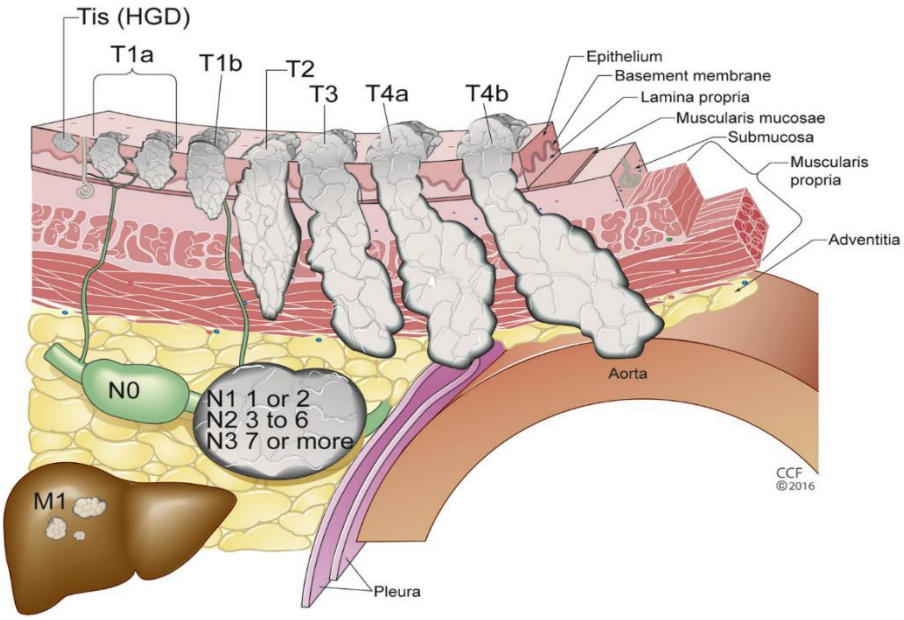


Figure 1.4.1: TNM staging of oesophageal cancer – American Joint Committee of Cancer AJCC 8th Edition

Illustrates the T stage which denotes the depth of tumour invasion locally. The N stage categorises the nodal status. Endoscopic ultrasound in conjunction with computer topography is used to the evaluate the T and N stage. The M stage denotes the presence of distant metastases. Figure from the publication by Ahmed et al (2010)(16)

Cardio-pulmonary exercise testing (CPEX) is evaluated a patient's cardiac and pulmonary and his or her ability withstand the stress of major surgery. This information provides the clinician with an objective assessment of a patient's fitness (17).

1.4.2 Curative Treatment

Surgery remains the mainstay for curative treatment of oesophageal cancer (18). However, over the last decade, advances in endoscopic technologies have led to the development of techniques of limited endoscopic mucosal resection (EMR) of early oesophageal cancer. EMR enables the clinician to perform a localised resection of the epithelium and mucosa, as is the case for Tis and T1a disease (19, 20). This procedure is performed via the endoscope with the patient under sedation and offers a potentially curative resection if the lesion is isolated to the mucosal region (20). Extension of the tumour into the submucosa as is the case in T2 and T3

disease, increases the likelihood of nodal spread and these patients would be considered for an oesophagectomy (21, 22).

Clinical trials have shown survival benefits of peri-operative treatment for patients undergoing curative treatment for locally advanced oesophageal cancer (3, 23, 24). At present, patients with evidence of nodal disease (N+) and/or tumour stage T3 or greater are considered for peri-operative treatment. The peri-operative chemotherapy regimens favoured in the UK are based on the outcomes of two randomised control trials. Results from the OEO5 trial showed improved rates of complete pathological response and a longer interval to disease recurrence in patients who received epirubicin, cisplatin and capecitabine (ECX)(25). The previous standard of care for patients undergoing peri-operative chemotherapy for oesopahgogastric cancer was the ECX regimen, which consists of three peri-operative and three post-operative cycles of the chemotherapy. The recently concluded FLOT-4 trial has showed higher complete pathological response rates of the primary tumour in patients who received docetaxel, oxaliplatin, 5-fluorouracil and leucovorin (FLOT) regimen when compared to the ECX regimen(24). As a consequence, the new standard of care in patients undergoing peri-operative chemotherapy for oesophagogastric cancer is the FLOT regimen which consists of four peri-operative and four post-operative cycles of the chemotherapy. The optimum peri-operative treatment modality remains unclear. The current Neo-AGIS trial is evaluating the role of chemo-radiotherapy versus chemotherapy alone in patients undergoing curative treatment for junctional adenocarcinoma (26). Peri-operative treatment is associated with risks. Peri-operative treatment has associated an increase in postoperative mortality and morbidity (27, 28). In addition, studies have shown that peri-operative chemotherapy has a detrimental effect on cardiovascular and respiratory function(17).

Following peri-operative treatment, patients have a repeat CT scan to evaluate the progression of the disease. If the disease remains curative, patients progress to surgery approximately six weeks after their last chemotherapy treatment.

The principles of an oncological resection involve resection of the primary tumour with adequate resection margins and a formal lymphadenectomy which involves the resection of the local lymph nodes that drain the primary tumour (18). Sub-total oesophagectomy involves resection of the mid and distal oesophagus and reconstruction with a gastric conduit. This

operation is performed with a midline incision in the abdomen and an incision in the right postero-lateral chest wall. Recent advances in technology have led to the development of the minimally invasive approach for oesophagectomy (MIO)(29-31). This involves key hole surgery in the chest to resect the oesophagus and perform an anastomosis in the chest or the neck, a procedure known as thoracoscopic oesophagectomy. The efficacy of the technique and the potential benefits are being assessed in randomised control trials(30-32). A potential benefit from the reduced handling of the primary tumour with the thoracosopic compare to open technique. Wang et al, observed a significant reduction in the number of CTCs in blood sample taken after surgery in their thoracoscopic versus the open oesophagectomy cohort, thus raising the possibility that tumour cell shedding is reduced in the thoracoscopic cohort (33).

Surgery is associated with a mortality of 2-4% and morbidity of 30-40% (34-36). Furthermore, there is a significant negative impact in the quality of life in patients post oesophagectomy (37, 38). Recent advances in the enhanced recovery pathway have led to a reduction in the recovery time and improved post-operative outcomes (39).

The response to peri-operative chemotherapy can be evaluated using the Mandard tumour regression grading system(40). Table 1.4.2.1 illustrates the tumour regression grade based on the response to peri-operative chemotherapy. Tumour regression grade has been shown to be an independent prognostic factors in patients who have underwent curative resection following peri-operative chemotherapy.

Figure 1.4.2.1 Mandard tumour regression Grade 1 to 5

Grade	Definition
TRG 1	No residual cancer
TRG 2	Rare residual cancer cells
TRG 3	Fibrosis outgrowing residual cancer
TRG 4	Residual cancer outgrowing fibrosis

TRG 5	Absence of regressive changes

Despite advances in peri-operative treatment and surgical technique, recurrence rate with curative intent remain at 46-53% with a median time to recurrence of 11-13 months (41-43). As a consequence, five year survival remains poor at 34-46% (44, 45). The prognostic factors that predict survival following resection are the final pathological stage of the tumour, the presence of nodal disease (N+) and the presence of lymphovascular and peri-neural invasion (44, 46).

The pattern of recurrence is classified based on the site of the primary recurrence detected radiologically. If the disease recurs at the site of the previous operation either in the chest or the abdomen, it is classified as locoregional recurrence. This encompasses both recurrence of the disease in the gastric conduit or the native oesophagus and/or the regional lymph nodes and/or the pleural/peritoneal cavity. Recurrent disease can present with malignant ascites or pleural effusion secondary to transcoeloeimc spread of the disease. Presence of metastases at distant sites, eg liver, brain is classified as distant recurrence and is attributed to haematogenous spread of the tumour cells from the primary site.

Studies that have evaluated the pattern of recurrence following curative treatment report varying distributions. Dresner et al, identified a higher rate of locoregional recurrence in comparison to distant recurrence in patients who underwent radical oesophagectomy with two field *lymphadenectomy*, 27% versus 18%(41). The majority of the patients in this study did not receive peri-operative chemotherapy. Parry et al, evaluated the outcomes of 171 patients who developed recurrence following curative treatment for oesophagogastric cancer, of whom 59% received peri-operative chemotherapy and/or radiotherapy and surgery (47). In their study, 76 patients (44%) developed distant recurrence with the liver and lung being the most common sites of metastases. Notably, over 122 patients (71%) had evidence of two or more sites of recurrence and 68 patients (40%) had evidence of both locoregional and distant recurrence. The study concluded that the presence of distant recurrence and the presence of three or more sites of recurrence were independant prognostic factors associated with recurrence free survival. Of the patients who relapsed, 62 (37%) received treatment for their

recurrence of which the majority had either locoregional (19) or solitary recurrence (24). Median post-recurrence survival was higher in the treatment group versus the best supportive care group, 9 months versus 2 months. However, the reason that the majority of patients received best supportive care was that they were not fit enough for any treatment. Their reduced fitness may explain or contribute to their reduced survival. A subgoup of patients underwent surgical resection of oligometastases (13), of whom 5 patients had resection of a solitary metastases from the brain. Seven of these patients died during the follow up period due to disease progression with a median post-recurrence survival of 11 months. Blom et al, noted lower rates of locoregional recurrence in patients who received peri-operative chemotherapy versus patients who underwent surgery alone for oesophageal cancer, 6 vs 16% (p=0.017)(48).

In all three studies, there was no defined protocol for radiological or endoscopic follow up of patients following curative resection for oesophagogastric cancer. Diagnostic imaging occurred only if there was clinical suspicion of recurrence based upon the patient's clinical signs and symptoms. Thus, the time between the development of the macroscopic presence of recurrence and the subsequent development of patient's symptoms are unknown. Definitive conclusions about the timing and pattern of recurrence following curative resection for oesophagogastric cancer remain unclear.

1.4.3 Palliative Treatment

Due to the nature of the disease, approximately 50% of patients with oesophageal adenocarcinoma present with advanced disease (49). Advanced disease is classified as disease that is not amenable to resection, due to the presence of disease invading adjacent organs or the presence of disease outside the resection field. The extent of disease can vary from a single peritoneal nodule to disseminated disease afflicting distant organs, eg liver. The focus of palliative treatment is to treat both the primary tumour and distant metastatic disease. Chemotherapy remains the mainstay for treatment of metastatic oesophageal adenocarcinoma (50). The monoclonal antibody, trastuzamb has been shown to improve survival in patients with HER2 positive adenocarcinomas of the oesophagogastric junction (51).

Dysphagia a common symptom in patients with locally advanced oesophageal cancer. Primarily attributed to the tumour causing either luminal or extraluminal obstruction. Various treatment

modalities are available to manage these symptoms. Radiotherapy and brachytherapy can be used to reduce the tumour burden and provide symptomatic relief(52, 53). Oesophageal stents can also be used to preserve the oesophageal lumen and thus allow patients to maintain enteral nutrition(53, 54).

1.5 Disseminated and Circulating Tumour Cells

Dissemination of tumour cells from the primary tumour can occur via four routes. Haematogenous dissemination is dissemination directly into the blood stream via the venous drainage from the primary tumour and lymphatic dissemination is dissemination via the lymphatic drainage channels. Alternatively tumour cells may infiltrate directly into organs adjacent to the primary tumour or transcoelomic dissemination by seeding of tumour cells within the pleural, pericardial, abdominal or pelvic cavity.

The reasons how and why cancers metastasise preferentially to specific sites within the body remains unclear. The concept of circulating tumour cells (CTCs) was first described by Thomas Ashworth in 1869 (55). During an autopsy on a patient with metastatic cancer, he noted that the presence of cells in the blood which were morphologically similar to those of the primary tumour. In 1889, Paget noted the preferential site of cancer metastases associated with different epithelial tumours. During autopsies of patients with breast cancer it was observed that metastases were present in the lung and liver (56). In contrast in patients with uterine cancer, distant metastases were not present at these sites. Paget concluded that the site of metastases was linked to the biological origin of the tumour and proposed the "seed and soil" hypothesis. He described the dissemination of cancer cells from the primary tumour as a seed which is then deposited at distant sites in the body. Subsequent development of the metastases at these sites was thought to be dependent upon the environment, the soil which is conducive to the growth. This early observation presaged ideas about the variation in the molecular nature of cancer metastases which is thought to be dependent upon the primary tumour from which the cells originate. An alternative model based upon anatomical dissemination was proposed by Ewing (57). It is accepted that both molecular characteristics and

anatomical location of the primary tumour contribute to determine the eventual site of distant metastases.

Our knowledge of tumour dissemination has evolved gradually since this early observation. Tumour dissemination and subsequent development of metastases were thought to be late occurrences in the evolution of the disease. However, 50% of patients undergoing curative resection for oesophageal adenocarcinoma develop recurrence within 5 years, with a median time to recurrence of 13 months (43, 58). This observation suggests that tumour dissemination to distant sites occurs at a far earlier stage in the development of the primary disease than surmised previously. This realisation has led to the development of the evolutionary model of tumour growth and dissemination. This model infers that tumour dissemination occurs during the initial stage of tumour development, prior to the tumour being detected macroscopically. This theory is supported by the detection of micrometastases in lymph nodes of patients who have undergone curative resection for oesophageal cancer (59). The presence of these metastases was associated with poor prognosis even in patients who had no macroscopic disease identified in the lymph nodes following curative resection (44, 60-62).Figure 1.5.1 illustrates two hypothetical models of tumour dissemination (63).

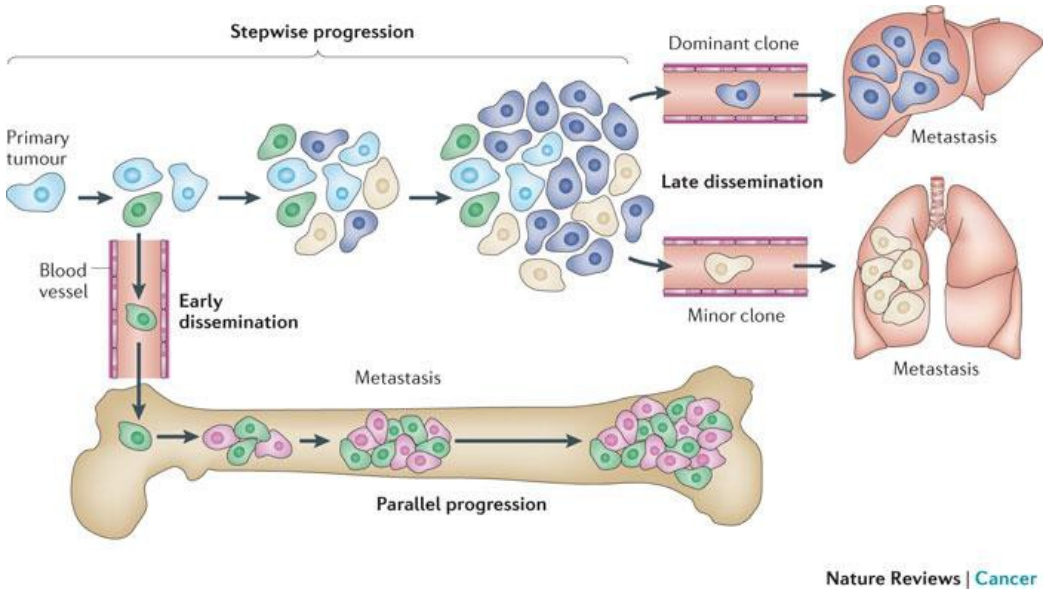


Figure 1.5.1: Models of tumour dissemination – Stepwise progression and parallel progression

The figure illustrates the two hypothetical models of tumour dissemination. The first model, parallel progression hypothesizes dissemination of the tumour cells during the early development of the primary tumour. Early dissemination of the cancer occurs before the primary tumour is detected macroscopically. The tumour cells proliferate at the distant sites, eg bone marrow. The disseminated cells are hypothesized to be clones that arose from the primary tumour. They may have evolved and hence many not be sensitive to the same chemotherapy agents as the primary tumour.

This model could explain the observed recurrence and relapse of patients who have undergone curative resection for cancer. The second model describes stepwise progression. In this model, dissemination of the cancer is at a late stage of tumour development. It occurs after the tumour is detectable macroscopically. There is clonal diversity between the cells of the primary tumour and tumour deposits at metastatic sites. This diversity could explain the varied response of the primary tumour and tumour deposits at metastatic sites to chemotherapeutic agents in patients with metastatic oesophageal cancer. Figures taken from publication by Maruysk et al (2012)

1.6 Circulating tumour cells

Circulating tumour cells (CTCs) were first detected and characterised in peripheral blood by Racila et al in 1998 (64). They isolated CTCs based upon the positive expression of epithelial cell adhesion molecule (EpCAM) and negative expression of, CD45 a leucocyte marker by immmunomagentic separation. Further characterisation of the cells by immunocytochemistry with a pan cytokeratin antibody against cytokeratins 5, 6, 8 and 18 indicated that 29 out of 30 breast cancer patients and all 3 prostate cancer patients had CTCs in the blood. A significantly higher number of CTCs were detected in the blood of patients with metastatic than in those with localised disease was noted, 122 ± 140 CTCs in patients with distant metastases versus 15.9 ± 17.4 in patients with disease confined to the organ (P=<0.001)(64). Even in the advanced patients, CTCs only accounted for a small proportion of the cellular composition of the blood. Presence of CTCs has been associated with poor prognosis in patients with breast, colorectal and prostate cancer (65, 66). The number of CTCs deemed to be of prognostic significance is variable. Cristofonnalli et al, concluded that >5 CTCs per 7.5 ml of blood was a significant prognostic factor in survival in patients with metastatic breast cancer (66). Various methods and technologies have been developed to enrich and detect CTCs from peripheral blood. The major challenge in this process is that CTCs occur at very low concentrations in the peripheral blood, ranging between 1–10 cells per 10 ml blood of most cancer patients in which CTCs are detected (67). The half-life of CTCs in the peripheral bloodstream was has been estimated to be approximately 1-2 hours (67). Further, there is a lack of a clear definition with

which to define a CTC. The only FDA approved method for the detection of CTCs is the CELLSEARCH® system (65). The system classifies a cell in the patients' blood as a CTC if it expresses the epithelial markers, EPCAM and cytokeratins 8, 18 or 19 and does not express CD45. However, studies have shown that there is heterogeneity within the CTC population and the expression of epithelial markers is variable (68). Of interest are the CTCs that have undergone or undergoing EMT within the bloodstream (69-74). These cells express some mesenchymal biomarkers and lose expression of epithelial markers. The reliance upon epithelial makers alone to detect and isolate CTC may significantly underestimate the population as demonstrated by Cristofanailli, who noted that only 50% of patients with metastatic breast cancer had CTCs (66). However, mesenchymal markers can also be expressed in cells of haematopoeitic lineage which do not disseminate (75). Estimation of the true number of CTCs that are undergoing EMT or not is challenging in the absence of a perfect and universal CTC biomarker marker is difficult.

Current enrichment and detection methods rely upon the unique physical and biological properties of CTCs to differentiate them from haematopoietic cells (67). CTCs can be isolated based upon their larger size, CTCs are typically >8 μm in diameter and that they are less deformable than haematopoietic cell (67). Several methods for the enrichment of CTCs based on their physical characteristics have been described (76). Membrane filtration system isolate CTCs based on size and CTC chip have been designed to isolate CTCs based upon their size and reduced deformability(77). Ficoll hypaque density centrifugation can separate CTCs based on the higher density of CTCs in comparison to haematopoietic cells (78). CTCs can also be isolated based on their electrical charge via a process called Dielectrophoresis (DEP)(79). A spiral CTC chip is used to separate CTCs based on the higher centrifugal forces applied to the larger CTCs in comparison to the smaller haematopoietic cells (80, 81).

Enrichment based upon the biological properties relies upon the biomarker expression of CTCs. Positive selection of CTCs has usually been with antibodies specific to epithelial markers eg EpCAM, cytokeratins. Of interest is the isolation of CTCs that show epithelial mesenchymal plasticity (EMP) and express mesenchymal biomarkers eg: vimentin (82). Unlike some other solid epithelial tumours, there is a lack of a tumour specific marker for oesophageal cancer. Potential biomarkers evaluated in oesophageal cancer are trefoil proteins, tyrosine kinase receptors and chemokine receptors. Trefoil proteins secreted from columnar mucosae of

the gastrointestinal tract and play a role in maintaining the integrity of the epithelium. They are under investigation as part of the BEST2 and BEST3 trial in the detection of Barrett's oesophagus (83). Human epidermal growth factor receptor 2 (HER2), Fibroblast growth factor 2 receptor (FGFR2) and tyrosine kinase cell surface protein receptor (c-Met) play a role in regulating normal cellular processes. These receptors are now potential therapeutic targets for intervention in the treatment for oesophageal carcinoma (84-86). Trastuzumab, a monoclonal antibody against HER2 in combination with chemotherapy has been shown to improve survival in patients with HER2 positive oesophageal junctional and gastric adenocarcinoma (51). Chemokine receptors CXCR4 and CCR7 are proposed to play a role in the metastatic dissemination of cancers(87). Kaifi et al, detected CXCR4 in 75 out of 136 patients who underwent curative resection for oesophageal cancer. CXCR4 expression was associated with a significant reduction in overall survival, 25 months in CXCR-4 positive patients versus 97 months in CXCR4 negative patients (88). Positive selection of white blood cells based upon their expression of leucocyte specific markers eg CD 45 or CD 44 provides an effective enrichment of CTCs that does not rely upon expression of any specific biomarkers by the CTCs (89, 90). Subsequently analyses of the biomarker expression is possible by immunocytochemistry and flow cytometry.

RNA based technologies, eg reverse transcription quantitative polymerase chain reaction (RT-qPCR) allows the amplification, detection and quantification of multiple transcripts within a sample (91). This method provides a surrogate measure of CTCs in a sample and analysis of small numbers of CTCs within a sample. However information about the physical characteristics and the specific numbers of CTCs are not available with this approach.

The EPISPOT technology, CTCs are enriched by positive immune-depletion of blood cells and placed on a membrane coated with an antibody for a tumour specific protein that is anticipated to be secreted or release by the CTCs (77)(92). Any tumour specific protein capture by the antibody with which the membrane was coated is detected with a second fluorescence conjugated antibody against the tumour specific protein. The fluorescence is detected by fluorescent microscopy. The viability of CTCs and their metastatic potential has been evaluated with xenotransplantation models, which involves the injection of CTCs(67).

The ideal method for detection and characterisation of CTCs would be one that is sensitive, reliable, reproducible and cost-effective. The lack of tumour specific markers for oesophageal cancer and the presence of heterogeneity within the CTC population remains a challenge. Analyses of a wide range of biomarkers should maximise the detection and characterisation of CTCs in oesophageal carcinoma patients.

1.7 Epithelial to mesenchymal plasiticity in oesophageal cancer

A current theory is that CTCs originate from a subpopulation of cancer stem cells within the primary tumour (93). The cancer stem cells are non-proliferative and thus resistant to the effects of chemotherapeutic agents (93, 94). The cancer stem cells undergo a transition from an epithelial to a more mesenchymal phenotype which allows the cells to enter the systemic circulation and travel to distant sites within the body. Figure 1.7.1 illustrates the factors that influence epithelial cells to undergo transition to mesenchymal cells (95).

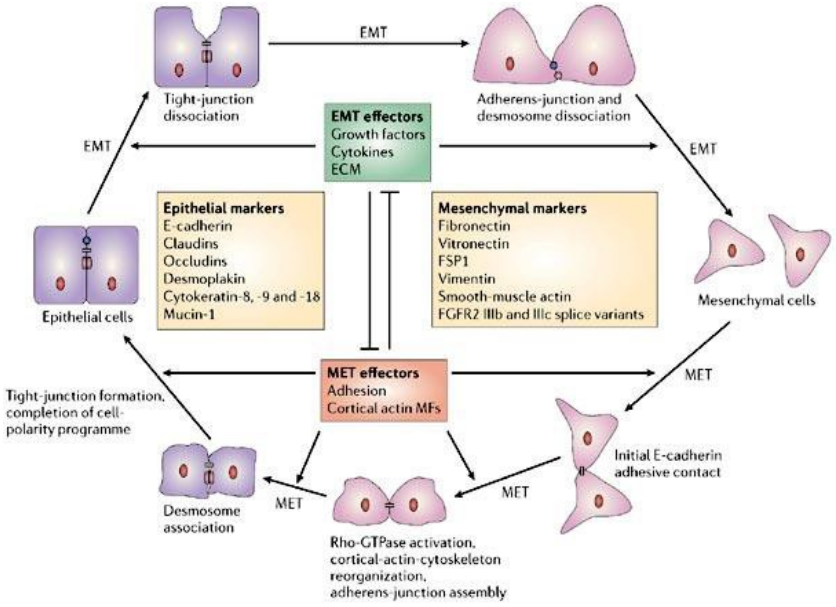


Fig 1.7.1: Interaction between epithelial markers, mesenchymal markers and transcription factors during epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET)

Epithelial cells lose their adherence to adjacent cells and basement membrane and undergoes a transition to a mesenchymal cell which is then able to enter the systemic circulation. Complex interaction between growth factors, cytokines and the extracellular matrix plays a role in this process. Figure taken from the publication Thiery at al (2006) (91)

Studies have shown that increased expression of the regulators of EMT, such as Twist 1, Zeb1, Zeb2, Snail1 and Snail2/Slug, in primary tumours is associated with an increased risk of metastatic relapse (96). Increased expression of these factors has been associated with poor prognosis in patients with oesophageal cancer (74). The presence of CTCs with mesenchymal phenotypes has been associated with poor prognosis in patients with breast and prostate cancer (97, 98).

Figure 1.7.2 illustrates the prognostic significance of epidermal growth factor (EGFR) expression in resected oesophageal cancer specimen (72)

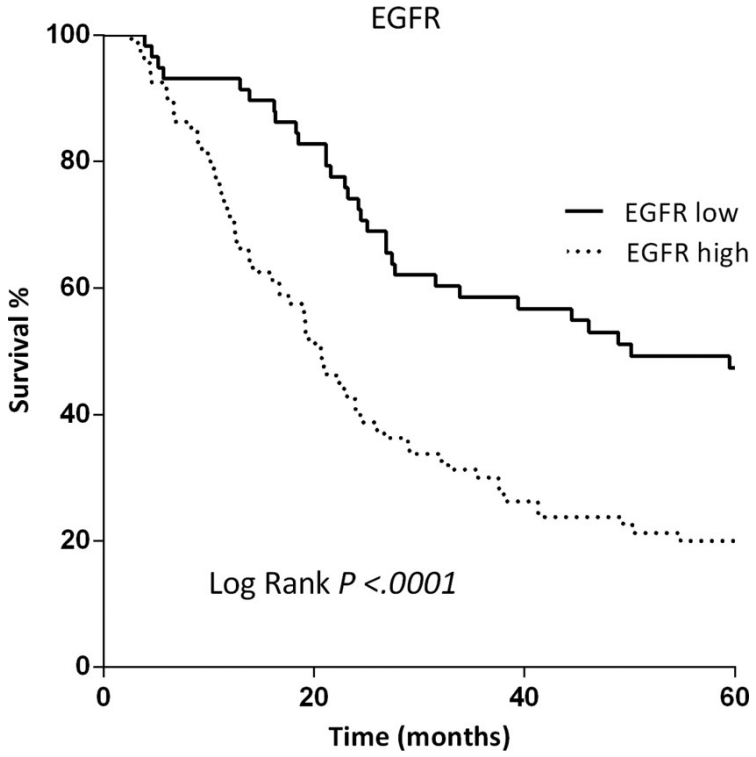


Figure 1.7.2: The prognostic significance of epidermal growth factor receptor (EGFR) expression in resected oesophageal cancer specimens

Higher EGFR expression was associated with a significant reduction in survival. Median survival was 59.5 months in patients with low EGFR expression and 20.8 months in patients with high EGFR expression ($p<0.001$, log rank). Figure taken from the following reference, Prins et al (2015) (68)

During the initial transition from an epithelial phenotype to a more mesenchymal phenotype, it is thought that the cells lose their adherence to surrounding cells and the basement membrane. E-cadherin and β-catenin are proteins that play a role in cell adhesion. It has been shown that the loss of E-cadherin expression in the primary tumours is associated with poor prognosis in patients undergoing curative surgery for oesophageal cancer (99). Epidermal growth factor receptor (EGFR) is a transmembrane protein that plays a role in regulating DNA synthesis and cell proliferation. Overexpression of EGFR has been associated with significantly worse prognosis in patients following curative resection for oesophageal adenocarcinoma (72).

During EMT, cells lose expression of epithelial markers. As a consequence, detection of these cells after this period of transition requires other biomarkers such as mesenchymal markers. Vimentin is a protein present in the cystoskeleton of mesenchymal cells. The presence of vimentin in the primary tumour of patients undergoing curative treatment for oesophageal cancer has been shown to be associated with poor prognosis (100).

1.8 Disseminated tumour cells in oesophageal adenocarcinoma

Bone marrow consists primarily of stroma, fibroblasts, macrophages and osteoblasts which form the framework and structure of the marrow. The cellular component comprises cells of haematopoietic origin, myelopoietic origin and erythropoietic origin that are interspersed within the stroma. Abundant blood supply and favourable microenvironment are thought to be among the reasons why tumour cells migrate to and reside in the bone marrow (101). The bone marrow has been proposed to act as a reservoir for metastatic tumour cells that can recirculate into the bloodstream and then to distant sites within the body (101). The presence of disseminated tumour cells within the bone marrow (BMTCs) has been associated with

poor prognosis in patients with breast, prostate and colorectal cancer (102-105). The presence of BMTCs has been linked to an increased risk of metastatic relapse in patients who have undergone curative treatment for breast cancer (106).

Fifteen studies have evaluated the role of BMTCs in patients undergoing curative treatment for oesophageal cancer (58, 107-120). The detection rates varied significantly between the studies and ranged from 32% to 88%. The majority of the studies were focused on patients with squamous cell carcinomas and only five of the studies included patients with oesophageal adenocarcinoma. Furthermore, the site of bone marrow sampling and the volumes of bone marrow obtained varied. Bone marrow was sampled from two primary sites. Bone marrow was extracted directly from the rib portion which is excised as part of thoracotomy during the oesophagectomy or from a bone marrow aspirate taken during surgery. The other site of bone marrow aspiration was the iliac crest. One study that compared sampling sites, noted that a significantly higher volume of bone marrow was obtained from the rib section compared to bone marrow aspirates from the iliac crest (114). The tumour cell detection rate in the rib section was noted to be 88% in comparison to 15% in the iliac crest(114). Methods for detecting BMTCs varied. The majority of studies placed bone marrow extracts onto glass plates and analysed expression of epithelial biomarkers by BMTCs, primarily EpCAM and/or cytokeratins.

Only a small proportion of the studies evaluated the role of peri-operative treatment on disseminated tumour cells. Ryan et al, detected significantly fewer BMTCs in patients who underwent neoadjuvant chemoradiotherapy in 12 out of 47 patients (39%) versus surgery alone in 27 out of 41 patients (61%) for oesophageal adenocarcinoma (116). There was no significant difference in patients who underwent peri- operative treatment for squamous cell cancer versus surgery alone. In contrast, O'sullivan et al showed no difference in the number of BMTCs in patients who underwent neo-adjuvant treatment and surgery versus surgery alone for oesophageal adenocarcinoma, however only 5 out of the 31 patients with adenocarcinoma underwent neo-adjuvant chemotherapy in this study (114). The authors concluded that there were chemoresistant cells within the population of BMTCs. The small subset of patients within the studies makes it difficult to assess confidently the impact of neo-adjuvant treatment on BMTCs in oesophageal cancer. The prognostic significance of disseminated tumour cells in oesophageal adenocarcinoma remains unclear. The follow up

times for the studies ranged from 20-60 months. The studies concluded that the presence of BMTCs was an independent prognostic factor in poor survival but there was a lack of consensus on the number of BMTCs deemed to be of prognostic significance. The primary reason for this issue maybe the significant variation between the methods for detecting BMTCs.

Two studies which followed patients over a 10 year period had different conclusions on the prognostic significance of BMTCs (110, 116). Gray et al, concluded that the presence of BMTCs was associated with adverse tumour characteristics but did not impact on long term survival or recurrence (110). Ryan et al, established that the presence of BMTCs was associated with increased risk of cancer-related death and was an independent prognostic factor in patients undergoing surgery alone for oesophageal cancer (116).

1.9 Circulating tumour cells in oesophageal adenocarcinoma

The prognostic significance of CTCs in oesophageal adenocarcinoma remains unknown. The majority of studies that have evaluated the role of CTCs in oesophageal cancer have focused on patients with squamous cell carcinomas (121-127). They conclude that the presence of CTCs had a significant association with poor survival and suggested that the presence of CTCs has a negative effect on prognosis. Direct comparisons between the studies is difficult due to variations in the patient cohorts and the methods used in the detection and enumeration of CTCs. Detection rates of CTCs in peripheral blood of patients with squamous cell carcinomas varied between 18%-45% (121-126). In three studies with a total of 90 patients with locally advanced oesophageal adenocarcinoma, CTCs were evaluated by RT-qPCR (121, 125, 126). The studies are limited therefore because individual CTCs were not identified but rather survivin mRNA, ERCC1 mRNA and CEA mRNA were quantified. Detection rates varied between 59.7% to 61% (121, 125, 126). Investigators concluded that ERCC1 mRNA levels correlated positively with response to peri-operative chemoradiation and that a fall in survivn mRNA levels following resection was an indicator of completeness of surgical resection (121, 125, 126).

Five studies have evaluated the presence of CTCs in patients undergoing curative treatment oesophageal adenocarcinoma based on the expression of epithelial markers by CTCs

(128-132). Reeh et al, noted the presence of CTCs in 14 out of 68 patients undergoing curative resection for oesophageal adenocarcinoma. CTCs were detected with CellSearch^{R} system. The criteria for CTC identification after positive selection of EpCAM expression was the presence of a nucleus, positive cytokeratin expression, round or oval cell morphology and absence of CD45 expression. The investigators concluded that in patients with adenocarcinoma, large tumour size and an advanced pathological stage of the tumour were associated with a higher number of CTCs in the blood (p=0.0027)(129). The number of patients in which CTCs were detected was lower in the cohort with squamous carcinomas, 10% (3/29 patients) than in the adenocarcinoma cohort, 20.6% (14/68) (129). The investigators had a lower threshold for the detection of CTCs than in previous studies using the CellSearch^{R} system. One or more CTCs detected per 7.5 ml of blood was taken to be significant. Two year follow up indicated that the presence of CTCs was an independent and strong prognostic factor for overall and recurrence-free survival in patients with oesophageal adenocarcinoma (129). The study is limited by the number of markers employed to characterise CTCs and dependence upon positive selection for EpCAM expression which means that the actual number of CTCs may be underestimated. Konzaella et al, evaluated the prognostic significance of CTCs and DTCs in the bone marrow in patients undergoing curative treatment for oesophageal adenocarcinoma. Bone marrow was aspirated from the iliac crest and cells were enriched by Ficoll density centrifugation and stained with pan-cytokeratin antibody(128). CTCs were enumerated from the blood using the CellSearch^{R} system. CTCs were detected in 20% (15/76) patients and DTCs in 17% (13/76) patients. Only three patients had both CTCs in the blood and DTCs in the bone marrow. The study concluded that CTCs were the only independent prognostic factor in relapse free and overall survival in patients undergoing curative oesophageal adenocarcinoma(128). Two other studies enriched CTCs from blood using filters which separated cells based on the size. Cells greater than 8 microns in diameter were isolated. Bobek et al, detected CTCs in 75% (12/20) patients using the MetaCell^{R} filter (130). Post enrichment, cells were identified based on a positive nuclear (NucBlue^{TM}) and cytoplasmic stain (CellTracker^{TM}). Kuvendjiska et al, reported on the sequential measurements of CTCs during the course of neo-adjuvant chemoradiotherpay and neo-adjuvant chemotherapy followed by surgery in patients undergoing curative treatment for oesophageal adenocarcinoma(132). CTCs were enriched from blood with the ScreenCell^{R} device which isolated cells based on side. Post enrichment, cells were labelled with pan-

cyokeratin and vimentin. Cells that expressed both biomarkers were classified as CTCs. In this study, CTCs were detected in 30% (6/20) patients prior to the start of treatment and the number of CTCs detected varied between 1-330 cells per 7ml of blood. Of the 20 patients in the study, 13 patients completed neo-adjuvant treatment and underwent surgery of which 5 patients had CTCs in the pre-treatment blood sample. The study reported that there was increase in the number of CTCs per ml of blood after neo-adjuvant therapy in these 5 patients. After completion of neo-adjuvant treatment and surgery, CTCs were detected in 62% (8/13) patients. The authors reported that 55% (5/9) patients who had CTCs at some time point during the course of treatment relapsed over a two year follow up period.

Dent et al, detected and characterised CTCs in patients with epithelial tumours by high resolution imaging flow cytometry (131). The criteria for CTC selection were on the presence of EpCAM expression and/or cytokeratins, presence of a nucleus and negative expression of CD 45. Two out of six patients (33%) with locally advanced oesophageal adenocarcinoma had CTCs and the numbers of CTCs ranged from 0 to 64 CTCs per 7.5 ml of blood.

1.10 Novel Biomarkers

In previous studies, CTCs were identified in patients with oesophageal adenocarcinoma based upon the presence of the epithelial biomarkers, EpCAM and/or cytokeratins. CTCs undergoing EMP may not express epithelial biomarkers. Incorporation of mesenchymal biomarkers, eg vimentin, could help identify this subgroup population of CTCs. Cadherins are types of cell adhesion molecules that regulate cell differentiation and maintains the structural integrity of epithelial cells. A feature of EMP is loss of E-cadherin expression and gain of N-cadherin expression(133). Reduced E-cadherin expression is hypothesised to play a role in loss of cellular adhesion and subsequent invasion and proliferation of tumour cells at distant sites. Reduced expression of E-cadherin in the primary tumour was associated with poor prognosis in patients with oesophageal cancer(134). Increased N-cadherin expression in oesophageal cancer is associated with an increase in the migratory and invasive capacity of the tumour cells present(135).

Identification of patients whose tumour cells are resistant to the chemotherapy regimes available before the onset of treatment would ensure that these patients proceed immediately to surgery. They would be spared the morbidity associated with peri-operative chemotherapy that would be in their case ineffective and the associated delay in their curative treatment. Platinum-based chemotherapeutic agents, eg cisplatin cause crosslinking of DNA strands, which leads to cell death. As part of DNA repair, the excision repair cross-complementing 1 (ERCC1) enzyme, which is involved in nucleotide excision repair pathway, excises platinum based adducts from the DNA strands. Studies have shown that high levels of ERCC1 expression in gastric and oesophageal cancer are associated with clinical resistance to platinum based chemotherapy agents(136). Fluorouracil (5-FU) or equivalent drugs are part of chemotherapy regimens for the treatment of oesophagogastric cancer. Their primary mechanism of action is to impair DNA replication by inhibition of thymidylate synthase. Thymidylate synthase is an enzyme responsible for the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) which is required for the synthesis of DNA and concomitant conversion of 5,10-methylenetetrahydrofolate to dihydrofolate. Increased expression of thymidylate synthase in the primary tumour has been associated with shorter disease free survival, poor tumour differentiation and resistance to chemotherapy in patients with gastric cancer(137).

HER 2 and cMET have been identified as potential therapeutic targets in patients with oesophagogastric cancer(51, 138). Trastuzumab, a monoclonal antibody that inhibits HER 2 activity improves survival in patients undergoing palliative treatment for gastric and oesophagogastric junctional tumours that express HER 2(51). Identification of CTCs which express these biomarkers in patients undergoing curative treatment could potentially indicate that these are appropriate therapeutic targets in the peri-operative and/or adjuvant setting.

The trefoil factor family of proteins are involved in mucosal protection and repair of mucosae of the gastrointestinal tract. Trefoil proteins are found in the malignant progression from Barret's oesophagus to adenocarcinoma(139). TFF3 is a trefoil protein that has been investigated as a potential diagnostic biomarker in Barret's metaplasia of the oesophagus(83).

As stated earlier, the tumour microenvironment consists of a heterogenous population of cells. Incorporation of a wide range of biomarkers, which includes epithelial, mesenchymal and therapeutic targets in the detection of CTCs will help characterise the heterogeneity of the

disseminated tumour cells and allow comparison with the primary tumour. These studies will help improve our understanding of molecular complexities of tumour metastasis, and could identify potential targets for therapeutic strategies or provide prognostic information.

Chapter 2: Materials and Methods

2.1 Laboratory Practice

Laboratory induction was completed as per the Newcastle University guidelines prior to commencement of the research at the Northern Institute of Cancer Research. Attendance at control of substance hazardous to health (COSHH) and biological safety risks assessment (BIOCOSHH) training was completed during the first month of the research study. The risk for each individual experiment was assessed and the summaries stored in the laboratory file. Laboratory coat and gloves were worn at all times during laboratory experiments.

2.2 Cell Culture

2.2.1 Culture of Oesophageal and gastric cancer cell lines

SK-GT-4, OE19, OE33, FLO-1 and ESO51 were cultured aseptically in a class II BioMat-2 microbiology safety cabinet (Medical Air Technology). Plates, flasks and pipettes for tissue culture were obtained from Corning. Cells were cultures in two different medium, Roswell Park Memorial Institute (RPMI) medium and Dulbecco's Modified Eagle's Medium (DMEM) (Table 2.2.1). To each 10% foetal calf serum (FCS) was added. Cells were incubated at 37^{o}C in a humidified environment with 5% CO_2 (Hereaus equipment). Cells were tested regularly to confirm the absence of mycoplasma and authenticity confirmed by Short Tandem analysis.

Table 2.2.1: Oesophageal adenocarcinoma cell lines and the corresponding culture medium

Cell Line	Origin	Catalogue Number	Medium*
SK-GT-4	Distal oesophageal adenocarcinoma	11012007	RPMI

FLO-1	Distal oesophageal adenocarcinoma	11012001	DMEM
OE19	Gastro-oesophageal junctional adenocaricnoma	96071721	RPMI
ESO51	Distal oesophageal adenocarcinoma	11012010	RPMI
OE33	Distal oesophageal adenocarcinoma	96070808	RPMI

* Roswell Park Memorial Institute (RPMI) medium and Dulbecco's Modified Eagle's Medium (DMEM)

Cells were analysed below passage number 50. Cell lines were thawed to room temperature and added to the appropriate media with 10% FCS. The cell suspension was transferred to a 25cm^3 Corning® flask that contained 20ml of media and placed in the incubator. Cells were divided once they had achieved 80% confluency into either a 75cm^3 flask or 125cm^3 flask dependent upon the number of cells required for the subsequent experiments.

2.2.1.1 Trypsinisation of cell lines for culture

Sterile phosphate buffered saline (PBS) (137 nM NaCl, 83 mM KCL, 10 nM Na_2HPO_4, ph 7.2) was prepared. Trypsin (Sigma Aldrich) was diluted to 10% with sterile PBS and stored at 4^{o}C.

The procedure for tyspsinisation of cell lines for culture was dependent upon the cell lines and the culture medium. ESO51 cell line were cultured in suspension with RPMI medium containing 10% FCS and 20mM L-glutamine. The cell suspension was aspirated into 20 ml sterile Falcon tube and centrifuged at 500g for 5 min. The cell pellet was resuspended in 5 ml RPMI containing 10% FCS. The cell suspension was then split into either a 75cm^3 **Corning®** flask or 125 cm^3 **Corning®** flask dependent upon the number of cells required for the subsequent experiments. For the remainder of the cell lines which grow as adherent cells, the medium was aspirated and discarded. The cells were washed twice with sterile PBS. Diluted trypsin was added to the flask, the volume was dependent upon the size of the flask. Cells were placed in trypsin for five minutes. After which an equivalent volume of full media was added to the flask. The cell suspension was aspirated and collected in a 15 ml Falcon centrifuge tube. The cell suspension was centrifuged at 500g for five minutes. The supernatant

was aspirated and discarded. If the cells were required for further culture they were resuspended in 5 ml of full media and divided into either a 75 cm^3 **Corning®** flask or 125 cm^3 **Corning®** flask.

Cell lines were fixed with 4% paraformaldehyde prior to use in the laboratory experiments. The cell pellet post trypsinisation was resuspended in 1 ml of 4% paraformaldehyde and incubated for 20 minutes to fix the cells prior to their use for laboratory. After 20 minutes, 10 ml of sterile PBS was added to the cell suspension and centrifuged at 500g for 5 minutes. The supernatant post centrifugation was discarded and the cell pellet resuspended in sterile PBS.

2.2.1.2 Freezing cell lines and bone marrow cells from the rib

Freezing media was prepared with the following constituents, 2.5 ml of sterile full media, 2.5ml of 20% FCS and 0.5 ml of 10% dimethylsulfoxide (DMSO). For cell lines in culture, the cell pellet post trypsination was resuspended in 2 ml of sterile freezing media. The cell suspension was transferred to a sterile cryovial and labelled with the details of the cell line, the passage and the date placed in storage. The cryovials were stored at - 80^0C.

Bone marrow solution was passed through a 70 micron filter and transferred to a sterile 20 ml Falcon tube. The solution was centrifuged at 500 g for 10 minutes. The cell pellet post centrifugation was resuspended in 2 ml of sterile freezing media. The cell suspension was transferred to a 2 ml sterile cryovial and stored at - 80^0C.

2.2.2 Culture of tumour cells from the exudates in patients with metastatic oesophagogastric cancer

Ascitic and/or pleural fluid samples were passed through a 70 micron filter. Post filtration, 50 ml of filtrate was transferred equally into two 50 ml Falcon tubes. The samples were centrifuged at 500g for 10 minutes. The supernatants were discarded. Twenty millilitres of the RPMI culture media listed in Table 2.2.2.1 were added to cell pellet in one of the 50 ml Falcon tubes. Twenty ml of the DMEM culture media listed in Table 2.2.2.1 was added to cell pellet in the other 50 ml Falcon tube.

Table 2.2.2.1 Culture medium used for the culture of tumour cells in the ascites

Patient	Volume of Ascites post filtration (ml)	Culture Medium *
P10	50	RPMI + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml) DMEM + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml)
JR	30	RPMI + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml)
SH1	50	RPMI + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml) DMEM + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml)
BB1	50	RPMI + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml) DMEM + 20% FCS + 1% Pencillin-Streptomycin (10 000 U/ml)

*RPMI (Rosewell Park Memorial Institute) (Herpes modification)(Sigma Aldrich), DMEM (Dulbecco's Modified Eagle medium)(Sigma Aldrich), FCS (foetal calf serum), Pencillin-Streptomycin (10 000 U/ml)(Gibco),

The cell pellets were resuspended in the culture media and placed directly into two individual 75 cm^2 Corning® flasks containing 20 ml of the corresponding culture medium. Cells were incubated at 37°C in humidified environment with 5% CO_2 (Hereaus Incubator). The culture media was changed at 24, 72 and 96 hours or until the cells reached 80% confluence. Cells were trypsinised as per the method described in Section 2.2.1.1.

2.2.3 Culture of oesophageal adenocarcinoma cells isolated from blood

Oesophageal adenocarcinoma cells were added to healthy volunteer blood to simulate the presence of circulating tumour cells in the blood. The following method describes the isolation of the oesophageal adenocarcinoma cells from blood directly into culture. The aim of the following experiment was to develop a protocol for isolating circulating tumour cells from the blood and disseminated tumour cells from the bone marrow directly into culture.

Two ml of blood obtained from a healthy volunteer. One hundred thousand SKGT-4 cells were added to the blood sample and placed in a 5 ml round bottomed polystyrene Falcon tube pre-blocked with 3% BSA solution. One hundred microlitres of Easy Sep™ Direct CTC enrichment cocktail (Catalog #19657) (50 μl/ml) was added to the blood sample and mixed gently. The suspension was incubated at room temperature for 5 minutes. The aliquot of Easy Sep™ RapidSpheres™ was vortexed gently. One hundred microliters of Easy Sep™ RapidSpheres™ was added to the cell suspension and mixed gently. The cell suspension with the Easy Sep™ RapidSpheres™ was incubated at room temperature for 10 minutes. The cell suspension was adjusted to a total volume of 5 ml with the addition of 3 ml of 3% BSA solution and placed in the Easy Sep™ magnet without a top at room temperature for 10 minutes. With the first polystyrene 5 ml Falcon tube still in the magnet, the magnet was inverted in one smooth clean movement over the second pre-blocked round bottomed polystyrene 5 ml Falcon tube for 2-3 seconds to decant the cell suspension. An additional 100 μl of Easy Sep™ RapidSpheres™ was added to the cell suspension and mixed gently. The cell suspension with the Easy Sep™ RapidSpheres™ was incubated at room temperature for 10 minutes. The cell suspension in the second tube was adjusted to a total volume of 5 ml with the addition of 3 ml of 3% BSA solution and placed in the Easy Sep™ magnet without a top at room temperature for 10 minutes. With the second polystyrene 5 ml Falcon tube still in the magnet, the magnet was inverted in one smooth clean movement over the third pre-blocked round bottomed polystyrene 5 ml Falcon tube for 2-3 seconds to decant the cell suspension.

The cell suspension post enrichment was resuspended in 20 ml of (RPMI and 20% FCS) and placed divided equally into two T75 cm^3 **Corning®** flasks. Cells were incubated at 37°C in humidified environment with 5% CO_2 (Hereaus incubator). The culture media was replaced at 24, 48 and 96 hours. The experiment was repeated with FLO-1 cells cultured in DMEM and 20% FCS.

2.3 Method for collection, storage and processing of volunteer and patient blood samples in preparation for imaging on the high resolution imaging flow cytometer and/or Fluorescence activated cell sorting (FACS)

2.3.1 Collection and storage of blood samples

Volunteer blood samples were obtained from the staff and students at the Northern Institute of Cancer Research (NICR) in accordance with the Ethics. Informed consent was obtained and a consent form was completed.

Patients were recruited into the study IRAS ID: 185350 in accordance with the NRES committee North East – Newcastle and North Tyneside 1 Ethics committee. Patients were recruited at two sites within the Newcastle Hospital NHS Trust: Royal Victoria Infirmary and Norther Centre for Cancer Care. Consent form (Appendix A) was completed. Blood samples were taken under aseptic conditions and collected in 6 ml tubes containing postassium ethylene-diaminetetraccetic acid (K_2EDTA) (BD Biosciences). The tubes were labelled with the corresponding patient identifier number. Samples taken within the Newcastle Hospital NHS Trust were transported to the NICR in a bag marked with the UN 3373 label (certified for transport of diagnostic human specimens) at room temperature. Blood sample were processed within four hours of collection.

2.3.2 Red cell lysis in blood

Five ml of 1.5 g bovine serum albumin diluted in sterile PBS (3% BSA:PBS) was added to three 50 ml Falcon tubes that were placed onto a bench-top roller at room temperature for 20 minutes. After 20 minutes, the 3% BSA solution was removed from the Falcon tubes and stored in a 14 ml Falcon tube for use later in the protocol. Two millilitres of blood was added to each of the three 50 ml Falcon tubes. Stock 20% Phospholysefix™ buffer solution was prepared in accordance with the manufacturer protocol. Two hundred millilitres of the solution was prepared with the addition of 128 ml of distilled water to 32 ml of the concentrated Phospholysefix™ solution at room temperature. The stock solution could be stored for up to one month at room temperature. The stock Phospholysefix™ buffer solution was placed in a water bath at 37°C for 30 minutes prior to red cell lysis. Forty millilitres of the stock Phospholysefix™ buffer solution pre-warmed at 37°C was added to each of the 50 ml

Falcon tubes. Each tube was inverted ten times to ensure adequate mixing of the Phospholysefix™ buffer solution with the blood. The samples were placed in a water bath at 37°C for 15 minutes. After 15 minutes, the samples were centrifuged at 500g for 10 minutes. The supernatant was poured off into a waste container in one smooth movement. Any excess lysis buffer was removed with a Pasteur pipette. One millilitre of 3% BSA was added to the cell pellet. Three 15 ml round-bottomed polystyrene Falcon tubes were pre-blocked with the addition of 2 ml of 3% BSA and placed on a bench-top roller for 20 minutes at room temperature. The 3% BSA solution was removed and discarded after this stage. One millilitre of 3% BSA was added to the cell pellet and the suspension was transferred into one 15 ml Falcon tube with a siliconised tip, 200 μl at a time. This process was repeated three times for each tube with 1 ml of 3% BSA to ensure the transfer of any residual cells into the one 15 ml Falcon tube. The cell pellet could be stored overnight at 4°C at this point.

2.3.3 Depletion of white blood cells

The cell suspension in the 15 ml round-bottomed polystyrene Falcon tubes was centrifuged at 500 g for 8 minutes. The supernatant is discarded post centrifugation. The next stage was the addition of 500 μl of 3% BSA solution using a siliconised pipette without resuspending the cell pellet. Twenty-five microliters of EasySep™ depletion cocktail (50 μl/ml) was added to the cell pellet and mixed gently with a siliconised pipette. The cell suspension was incubated at room temperature for 15 minutes. The aliquot with the EasySep™ magnetic beads was agitated gently and 50 μl of EasySep™ magnetic beads solution (100 μl/ml) was added. The cell suspension was incubated for 10 minutes at room temperature. The cell suspension was re-constituted to a total volume of 5 ml with the addition of 4.5 ml of 3% BSA solution. The cell suspension was mixed gently to prevent the magnetic beads from coalescing at the bottom of the tube. Five microliters was aspirated from the cell suspension and diluted 1 in 10 in PBS. The cells were counted with a haemocytometer.

The round bottomed 15 ml polystyrene Falcon tube containing the cell suspension was placed into the EasySep™ magnet without its cap and incubated for 10 minutes. With the first polystyrene 15 ml Falcon tube still in the magnet, the magnet was inverted in one smooth clean movement over the second pre-blocked round bottomed polystyrene 15 ml Falcon tube for 2-3 seconds to decant the cell suspension. The second tube was placed in the EasySep™

magnet and incubated at room temperature for 10 minutes. With the second polystyrene 15 ml falcon tube still in the magnet, the magnet was inverted in one smooth clean movement over the third pre-blocked round bottomed polystyrene 15 ml Falcon tube for 2-3 seconds to decant the cell suspension. The following additional step was included to improve the recovery of CD 45 negative cells. Five millilitres of 3% BSA:PBS solution was added to the first polystyrene 15 ml Falcon tube and re-inserted into the EasySep™ magnet 10 minutes at room temperature. With the first polystyrene 15 ml Falcon tube still in the magnet, the magnet was inverted in one smooth clean movement over the third Falcon tube containing the CD 45-depleted cell suspension.

2.3.4 Permeabilisation of cells

Permeabilisation solution was constituted with the addition of 5 ml of BD Perm/wash™ to 45 ml of sterile water. The BD Perm/wash™ solution was stored at 4°C. The solution was warmed to room temperature for 15 minutes prior to use.

The CD 45 depleted cell suspension in the 15 ml Falcon tube was centrifuged at 500g for 5 minutes. The supernatant was discarded. Two hundred microliters of the BD Perm/wash™ solution was added to the cell pellet and the cells were transferred into a 1.5 ml Eppendorf tube with a clean siliconized pipette. This process was repeated four times to ensure that all the cells have been transferred from the 15 ml Falcon tube into the Eppendorf. The cells were incubated at room temperature for 1 hour or overnight at 4°C.

2.3.5 Antibody labelling

The cell suspension with the BD Perm/wash™ solution post permeablisation was centrifuged at 500g for 5 minutes. The supernatant was removed and discarded. Three hundred microliters of the BD Perm/wash™ solution was added to the cell pellet in the Eppendorf and gently mixed with a siliconized pipette. The cell suspension was divided equally into three 1.5 ml Eppendorf tubes each containing 100 μl. Each Eppendorf tube is labelled with the corresponding antibody bodies in the three panels listed in Table 2.3.5.1, 2.3.5.2 and 2.3.5.3..

Table 2.3.5.1: Antibodies and the corresponding fluorochromes in panel A

Antigen	1° antibody	Fluorochrome	Volume in 100 µl of cell suspension
E-cadherin CD 324	Anti-human Biolegend #324110	Alexa Fluor® 488 conjugated	1 µl
Pan cytokeratin (clone C-11)	Cayman Chemical #10478	PE conjugated	3 µl
N-cadherin CD325	Anti-human Biolegend #350816	Alexa Fluor® 568 conjugated	1 µl
EpCAM CD326 (clone 9C4)	Mouse BioLegend #324214	PE-Cy5.5 conjugated	2 µl
CD45 (clone H130)	Mouse BioLegend #304016	PE-Cy7 conjugated	1 µl
DAPI	Bioloegend #422801	1 µl of 10 µg/ml	1 µl
Vimentin (V9)	Santa Cruz #sc-6260	Alexa Fluor® 647 conjugated	0.5 µl

Table 2.3.5.2: Antibodies and the corresponding fluorochromes in panel B

Antigen	1° antibody	Fluorochrome	Volume in 100 µl of cell suspension
c-MET (clone L6E7)	Cell Signalling Rabbit #8741S	Alexa Fluor® 488 conjugated	1 µl
Pan cytokeratin (clone C-11)	Cayman Chemical #10478	PE conjugated	3 µl
EpCAM CD326 (clone 9C4)	Mouse BioLegend #324214	PE-Cy5.5 conjugated	2 µl

HER2 CD340 (clone 24D2)	Mouse BioLegend #324413	PE-Cy7 conjugated	1 µl
DAPI	Bioloegend #422801	1 µl of 10 µg/ml	1 µl
CD 45 (clone H130)	Mouse Biolegend #304018	Alexa Fluor® 647 conjugated	0.5 µl

Table 2.3.5.3: Antibodies and the corresponding fluorochromes in panel C

Antigen	1° antibody	2° antibody	Fluorochrome	Volume in 100 µl of cell suspension
Thymidylate synthase (clone D5B3)	Rabbit Cell Signalling #9045S	Anti-rabbit AF488	Alexa Fluor® 488	1 µl
Pan cytokeratin (clone C-11)	Cayman Chemical #10478		PE conjugated	3 µl
ERCC1 (clone 4F9)	Mouse Dako #364829-2 – 1 ul	Anti-mouse AF 568	Alexa Fluor® 568	1 µl
EpCAM CD326 (clone 9C4)	Mouse BioLegend #324214		PE-Cy5.5 conjugated	2 µl
CD45 (clone H130)	Mouse BioLegend #304016		PE-Cy7 conjugated	1 µl

DAPI	Biolegend #422801			1 μl
TFF 3	2mg/ml		AF 647	1 μl

All of the antibodies in panels A and B were conjugated and did not require the addition of a secondary, conjugated antibody for detection. Conjugated antibodies listed in Tables 2.3.5.1 and 2.3.5.2 and 1 μl of DAPI at the following concentration 10 μg/ml were added to cells in the corresponding Eppendorf tubes. The antibodies were incubated for 30 minutes at room temperature in the dark. One millilitre of PBS was added to the cells in each Eppendorf tube and mixed gently. The cell suspension in each Eppendorf tube was centrifuged at 500 g for 5 minutes and the supernatant was discarded. The cell pellet in each Eppendorf tube was resuspended in 100-150 μl of PBS and the sample was ready for analysis on the high resolution imaging flow cytometer or by FACS.

Panel C contains two primary antibodies that were not conjugated. For Panel C, cells were incubated sequentially with these two primary antibodies and the with their secondary conjugated detection antibodies prior to the incubation of the other antibodies in the panel. The primary antibodies listed in Table 2.3.5.3 were added to the cell suspension at the required concentration to the Eppendorf labelled panel C and incubated at room temperature in the dark for 30 minutes. One millilitre of PBS was added to the Eppendorf and mixed gently. The cell suspension was centrifuged at 500 g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 100 μl Perm/wash™ solution. The secondary conjugated antibodies listed in table 2.3.5.3 were added to the cell suspension in the Eppendorf tube labelled panel C at the required concentration and incubated at room temperature in the dark for 30 minutes. One millilitre of PBS was added to the Eppendorf and mixed gently. The cell suspension was centrifuged at 500g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 100 μl Perm/wash™ solution. The conjugated antibodies listed in Table 2.3.5.3 and DAPI were added at the required concentration to the cells in the Eppendorf tube labelled panel C. Conjugated antibodies and DAPI listed in tables 2.3.5.1 and 2.3.5.2 are added to the corresponding Eppendorf tubes.

Figure 2.3.5.4 illustrates a flowchart of the protocol described above and a brief description of the mechanism of action of each reagent used.

Four millilitres of blood added to pre-blocked 50 ml Falcon tube with 3% BSA:PBS

BD Phospholyse fix buffer ™ is an aqueous buffered solution that contains formaldehyde and proprietary ingredients. The solution lyses red cells and fixes the remainder of the cells in whole blood

Addition of 46 ml of BD Phospholyse fix buffer ™. Sample inverted eight times and placed in water bath at 37ºC for 15 minutes. Sample centrifuged at 500g for 10 minutes

Supernatant discarded post centrifuge. Cell pellet resuspended in 1 ml of 3% BSA:PBS. Transfer of cell pellet with 200 µl polythene pipette tip into a pre-blocked 14 ml Falcon tube with 3% BSA. Additional 1ml of 3% BSA:PBS added to 50 ml Falcon tube and cell suspension transferred into 14 ml Falcon tube.

Cell suspension in 14ml falcon tube centrifuged at 500 g for 5 minutes. Supernatant discarded. Addition of 25 µl of EasySep™ Human CD45 Depletion Cocktail with a 200 µl polythene pipette tip to the cell pellet and incubation at room temperature for 5 minutes.

EasySep™ Human CD45 Depletion Cocktail contains antibody complexes which bind to CD45 positive cells within the suspension. A secondary part of the antibody complex is a site specific to the magnetic particles present in the EasySep™ Dextran RapidSpheres™

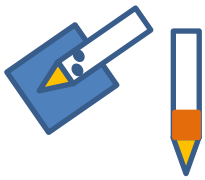
Addition of 50 µl EasySep™ Dextran RapidSpheres™ to the cell suspension and incubation at room temperature for 5 minutes.

Addition of 4.5ml 3% BSA:PBS to the cell suspension.

EasySep™ Dextran RapidSpheres™ are magnetic particles that bind the antibody complexes present in the EasySep™ Human CD45 Depletion Cocktail which are by now bound to the CD-45 positive cells in the suspension.

Cell suspension placed into EasySep™ magnet for 10 minutes. The 14 ml Falcon tube with the cell suspension in the EasySep™ magnet are held together and the cell suspension in transferred into a 14 ml Falcon tube pre-blocked with 3% BSA:PBS. The cell suspension in the 14 ml Falcon tube post immunomagnetic separation is placed into the EasySep™ magnet for 10 minutes. The process is repeated. The cell suspension post immunomagnetic separation is transferred into a 14 ml Falcon tube pre-blocked with 3% BSA:PBS

EasySep™ Dextran RapidSpheres™ magnetic particles which are indirectly bound to CD45 positive cells are separated from the cell suspension during this stage and become adherent to the sides of the Falcon tube.



The cell suspension in the 14 ml Falcon tube post-immunomagnetic separation is centrifuged at 500 g for five minutes. The supernatant is discarded post centrifugation. The cell pellet is resuspended in 1 ml 3% BSA:PBS and transferred into a 1.5ml Eppendorf tube with a 200 µl polythene pipette tip.

Cell suspension in Eppendorf tube centrifuged at 500 g for 5 minutes. Supernatant discarded. Addition of 100 µl 10% Permwash™ buffer. Sample incubated at room temperature for 30 minutes or overnight at 4ºC. Addition of 1 ml PBS and sample centrifuged at 500 g for 5 minutes. Supernatant aspirated and discarded. Cell suspension resuspended in 100 µl 10% Permwash™ buffer.

Addition of antibodies raised against intracellular proteins conjugated to fluorophores at required concentration. Eppendorf tube covered in foil and placed on rollers for 30 minutes.

Permwash™ contain feotal bovine serum and saponin. The solution acts to maintain the cells' structure, permeabilise the plasma membrane to allow antibodies to enter the cell for detection of intracellular

Addition of antibodies specific to cell membrane proteins conjugated to fluorophores and the nuclear dye at required concentrations. Incubation of the antibodies for 30 minutes with the Eppendorf tube covered

Post incubation, 1 ml PBS added to cell suspension and sample centrifuged at 500 g for 5 minutes. Supernatant aspirated and discarded. Cell pellet resuspended in 100 µl PBS. Sample is ready to be analysed by imaging flow cytometry or can be kept covered in foil overnight at 4ºC

Figure 2.3.5.4: Protocol for collection, storage and processing of volunteer and patient blood samples in preparation for high resolution imaging flow cytometry

2.4 Method for collection, storage and processing of bone marrow from discarded rib section in preparation for analysis by high resolution imaging flow cytometer

2.4.1 Collection and storage of bone marrow from the rib section

Patients were recruited into the study IRAS ID: 185350 in accordance with the NRES committee North East–Newcastle and North Tyneside 1 Ethics committee. Patients were recruited at the Royal Victoria Infirmary and consent form (Appendix B) was completed prior to surgery. The rib section was excised as part of a routine open thoracotomy during a two stage oesophagectomy. The excised rib section was collected in a sterile universal container and transferred to the NICR in a bag marked with the UN 3373 label (certified for transport of diagnostic human specimens) at room temperature. Samples were processed within four hours of collection. The rib section was broken in half with a bone cutter. The marrow was

recovered from the rib with toothed forceps and placed in 10 ml of sterile PBS. The solution was passed through a 70 micron filter into a pre-blocked 50 ml Falcon tube. Forty millilitres of the Phospholysefix^{TM} buffer solution pre-warmed at 37^{o}C was added. The tube was inverted ten times to ensure adequate mixing of the Phospholysefix^{TM} buffer solution with the bone marrow. The samples were placed in a water bath at 37^{o}C for 15 minutes. After 15 minutes, the samples were centrifuged at 500 g for 10 minutes. The supernatant was poured off into a waste container in one smooth movement. Any excess lysis buffer was removed with a Pasteur pipette. One millilitre of 3% BSA is added to the cell pellet.

For the first ten patients, twenty ml of the bone marrow suspension post filtration was obtained and divided into two 50 ml Falcon tubes. Cells from Ten ml of the bone marrow suspension were processed for CD 45 depletion and incubated with antibodies prior to analysis on high resolution imaging flow cytometer. The cell pellet from the second 10 ml of the bone marrow solution post red cell lysis was stored at -80^{o}C described in section 2.2.1.2.

2.4.2 CD 45 depletion and antibody labelling of cells in the rib bone marrow

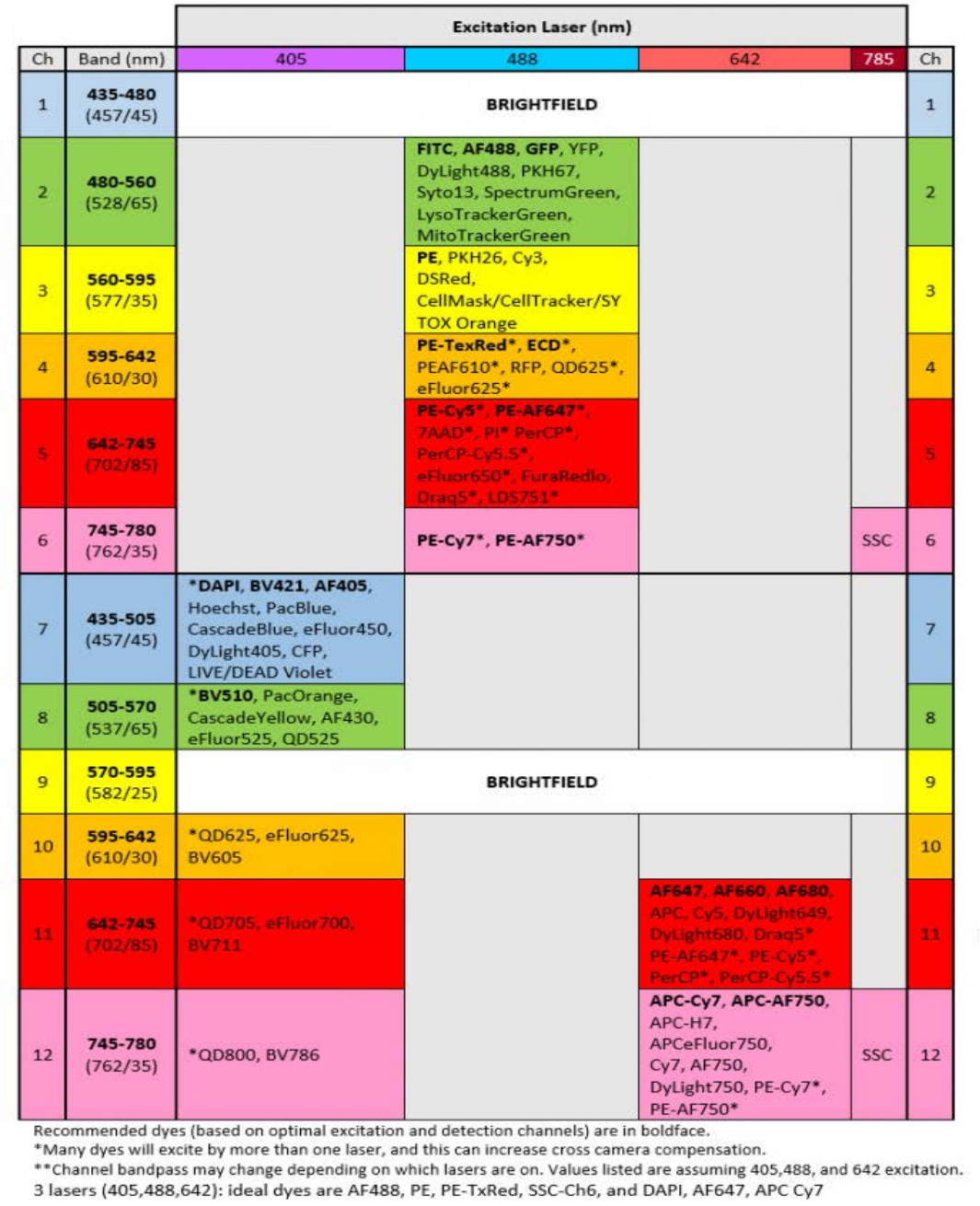
The cells suspension isolated from the bone marrow solution processed for CD 45 depletion as described in section 2.3.3. The method for permeabilisation and incubation with antibodies was described in Sections 2.3.4 and 2.3.5. Post CD 45 depletion, the cell suspension in the bone marrow was labelled with the antibodies in the three panels listed in Tables 2.3.5.1, 2.3.5.2 and 2.3.5.3.

2.5 Analysing cells on the high resolution imaging flow cytometer

2.5.1 Developing the compensation matrix

The Imagestream^{TM} imaging flow cytometer available in the NICR contains two high resolution cameras and four excitation lasers with wavelengths of 405 nm, 488 nm, 561 nm and 642 nm. There are 12 channels for detection of individual cells, two of which are the bright field channels that capture high resolution images of individual objects. The bright field images were captured routinely with a 40x objective. The remaining 10 channels are set at defined wavelengths and are available to detect fluorescence from fluorochromes conjugated to invidual antibodies following excitation by the lasers. The lasers were set at their maximum intensity. Each fluorochrome is assigned for detection in a specific channel based upon its

individual excitation and emission spectra. Figure 2.5.1.1 illustrates the excitation and the emission spectra of fluorochromes recommended by the suppliers of the Imagestream™ imaging flow cytometer (140).



		Excitation Laser (nm)				
Ch	Band (nm)	405	488	642	785	Ch
1	**435-480** (457/45)	**BRIGHTFIELD**				1
2	**480-560** (528/65)		**FITC**, **AF488**, **GFP**, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen			2
3	**560-595** (577/35)		**PE**, PKH26, Cy3, DSRed, CellMask/CellTracker/SYTOX Orange			3
4	**595-642** (610/30)		**PE-TexRed***, **ECD***, PEAF610*, RFP, QD625*, eFluor625*			4
5	**642-745** (702/85)		**PE-Cy5***, **PE-AF647***, 7AAD*, PI* PerCP*, PerCP-Cy5.5*, eFluor650*, FuraRedlo, Draq5*, LDS751*			5
6	**745-780** (762/35)		**PE-Cy7***, **PE-AF750***		SSC	6
7	**435-505** (457/45)	***DAPI**, **BV421**, **AF405**, Hoechst, PacBlue, CascadeBlue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet				7
8	**505-570** (537/65)	***BV510**, PacOrange, CascadeYellow, AF430, eFluor525, QD525				8
9	**570-595** (582/25)	**BRIGHTFIELD**				9
10	**595-642** (610/30)	*QD625, eFluor625, BV605				10
11	**642-745** (702/85)	*QD705, eFluor700, BV711		**AF647**, **AF660**, **AF680**, APC, Cy5, DyLight649, DyLight680, Draq5* PE-AF647*, PE-Cy5*, PerCP*, PerCP-Cy5.5*		11
12	**745-780** (762/35)	*QD800, BV786		**APC-Cy7**, **APC-AF750**, APC-H7, APCeFluor750, Cy7, AF750, DyLight750, PE-Cy7*, PE-AF750*	SSC	12

Recommended dyes (based on optimal excitation and detection channels) are in boldface.
*Many dyes will excite by more than one laser, and this can increase cross camera compensation.
**Channel bandpass may change depending on which lasers are on. Values listed are assuming 405,488, and 642 excitation.
3 lasers (405,488,642): ideal dyes are AF488, PE, PE-TxRed, SSC-Ch6, and DAPI, AF647, APC Cy7

Figure 2.5.1.1: Excitation and emission spectra of fluorochromes on the Imagestream™ machine (129)

Figure taken from https://ysm-websites4-live-prod.azureedge.net/immuno/flowcore/instrumentation/TACamnis_311819_39211_v2.jpg

UltraComp eBeads™ contain two population of spherical particles, one population can be coated with antibodies conjugated to individual fluorophores and the second population that will not bind to antibodies. Both populations were present when the UltraComp eBeads™ are used as single colour compensation controls. Twenty microliters of the beads were incubated with each conjugated antibody at the concentrations listed in Tables 2.3.5.1, 2.3.5.2 and 2.3.5.3. The brightfield channel was turned off and the laser settings were set at the maximum intensity. The machine was calibrated to calculate spectral overlap for each fluorochrome analysed. A compensation matrix (cif) which calculates the spectral overlap between the flurochromes was created for each panel of antibodies listed in 2.3.5.1, 2.3.5.2 and 2.3.5.3. The single colour controls and the compensation matrix (cif) was recalibrated each time when a new aliquot of antibody was purchased.

2.5.2 Gating template

The ability of the machine to capture high resolution images of individual cells means that large image files are generated which leads to an increase in the time taken to process an individual clinical sample. Consequently, further downstream processing and analysis of the image files was difficult. Inclusion of a gating strategy ensures that only cells of interest are captured which reduces the file sizes generate and allows more of clinical sample to be collected for analysis in each file.

Oesophageal cancer cell lines described in Table 2.2.1 were labelled with epithelial biomarkers (EpCAM and cytokeratins) and the therapeutic predictive biomarkers (HER2) and mixed with white blood cells from healthy human volunteer blood. Cells were labelled with antibodies as listed in Tables 2.3.5.1, 2.3.5.2 and 2.3.5.3. The cell lines and white blood cells were analysed by the high resolution imaging flow cytometry. The morphological characteristics of the oesophageal cancer cells and white blood cells, eg size, nuclear density, intensity of CD45 biomarker immunoreaction and a threshold value for each criteria was determined based on the data. The smallest oesophageal cancer cell line was OE19, individual OE19 cells were 10 μm in diameter. The smallest white blood cell was 8 μm in diameter. Based on previous studies, the smallest CTCs detected previously were 10 μm in diameter. The following gating template was applied to identify CTCs in the blood: diameter $\geq$ 10 μm and presence of a nucleus based on the intensity of DAPI. The same gating template was adapted for imaging

DTCs in the bone marrow. Bone marrow contained a large amount of white blood cells thus the gating template was as follows: diameter $\geq$ 10 μm, presence of a nucleus based on the intensity of DAPI and CD45 negative cells.

2.5.3 Analysis of image files

Each sample processed on the high resolution imaging flow cytometer was stored as a raw image file (rif). The IDEAS software combines the raw image file (rif) with the corresponding compensation matrix (cif), eg images of CTCs labelled with antibodies listed in panel A captured as raw image files were combined with the compensation matrix for the antibodies conjugated with the fluorophores in panel A. The software generated a data analysis file (daf). IDEAS software allowed further analysis of the data. Statistical analysis of the cell counts and data generated as described in Chapter 5 and 6 was with Prism Graph pad (version 8).

2.6 Fluorescence activated single cell sorting (FACS)

Cells were analysed by Fluorescence activated single cell sorting (FACS) with a BD FACSAria™ Fusion. This is a high speed flow cytometer that allows single cells to be isolated. Details of the machine are described in Chapter 4.

2.6.1 Sample analysis by high resolution imaging cytometry and confocal microscopy post Fluorescence activated single cell sorting (FACS)

Oesophageal cancer cell lines listed in section 2.2.1, OE19 and SK-GT-4 fixed in 4% paraformaldehyde and added to blood obtained from healthy human volunteer under the NICR ethics. Details of the experiments are described in Chapter 4. Red blood cells were lysed with Phospholysefix^{TM} buffer as described in Section 2.3.2. The cells were centrifuged and the cell pellet was resuspended in 1 ml PBS and transferred to a 6 ml FACS tube with a siliconized pipette. The cell suspension was incubated with the antibodies described in Chapter 4, Section 4.2 for 30 minutes. Two millilitres of PBS was added to the cell suspension in preparation for the FACS single sorting. The sample was covered in foil and transferred to the flow cytometry centre for sorting.

2.6.1 Culture of cells sorted by FACS

Oesophageal cancer cell lines were cultured as described in Section 2.2.3. Following trypsinisation, the cell suspension was counted with a haemocytometer. Blood obtained from healthy human volunteer under the NICR ethics and collected in an EDTA tube was transferred under aseptic conditions into a 15 ml Falcon tube. The required number of cells for the experiment was added directly under sterile conditions into the Falcon tube with the blood sample. BD Pharm Lyse™ stock solution was diluted with sterile water at a ratio of 1:10 to create a BD Pharm Lyse™ buffer. The BD Pharm Lyse™ solution lysed red blood cells without fixing the cells. BD Pharm Lyse™ buffer solution was warmed to room temperature was added to the blood at the following ratio: 10 ml of BD Pharm Lyse™ buffer solution per 1 ml of blood. The cells were vortexed gently for 30 seconds and incubated in the dark at room temperature for 15 minutes and centrifuged at 300 g for five minutes. The supernatant was aspirated and discarded. The cell pellet post red blood cell lysis was resuspended in 100 μl of sterile PBS and incubated with the antibodies listed in Section 4.4, (EpCAM, HER2, CD45) for 30 minutes in the dark. One millilitre of sterile PBS was added to the 15 ml Falcon tube and the cell suspension was transferred under sterile conditions into a 6 ml FACS tube with a siliconized pipette tip. The process was repeated with an additional two ml of sterile PBS. The sample was covered in foil and transferred to the flow cytometry centre for sorting.

2.6.2 Creation of a gating template

The staff at the single cell laboratory assisted in the processing of samples on the BD FACSAria™ Fusion cell sorter. UltraComp eBeads™ were used to create single cell controls for each fluropohore. The single colour controls were used to optimise the voltage settings of the lasers used in the FACS machine. Oesophageal cancer cell lines and white blood cells provided guides to create the gating template which would identify the single cells with a nucleus. The template ensured that only cells of interest would be captured.

2.6.3 Sorting single cells for culture by FACS

The compensation matrix was created based on the relevant fluorophores and the gating template was created. The sample was loaded into the machine. The nozzle size for the sorter was set at 100 μm. An initial 100 μl of the sample was processed in the machine. The gating

template as described in Section 2.6.2 was used to identify the populations of cells of interest which are then selected for collection. The cells were sorted into individual six well plates containing 2 ml of RPMI with 20% FCS.

2.6.4 Sorting single cells for imaging on the high resolution imaging flow cytometer by FACS

As described in Section 2.6.3, the compensation matrix and gating template were created for each experiment. Cells of interest were isolated and sorted into 1.5 ml Eppendorf tube. The Eppendorf tubes were covered in foil and were ready for processing on the high resolution imaging flow cytometer.

2.6.5 Sorting single cells for imaging on the confocal microscope with the FACS machine

As described in Section 2.6.3, a compensation matrix and gating template were created for each experiment. Cells of interest were isolated and sorted and placed directly onto a microscope slide. After the sorting process, the cells were secured with a cover slide. The cells were imaged by confocal microscopy.

2.7 Imaging cells on the confocal microscope

Images of cells post single cell sorting were captured with a Leica TCS SP2 UV AOBS point scanning confocal microscope. Confocal analyses were with assistance of the support staff Bioimaging unit, Faculty of Medical Sciences, Newcastle University.

2.7.1 Setting up the confocal microscope

Standard operating protocol for the Leica TCS SP2 UV AOBS confocal microscope was followed in preparation for imaging a sample.

2.7.2 Capturing images on the confocal microscope

The microscope slide was placed onto the microscope. A wide angle image of the slide was taken. The imaging software divides the image into 16 equal quadrants. Cells were identified based upon their nuclear stain and are marked in each quadrant. The lasers and filters are were calibrated for each individual fluorophore and an image of the cells at 40 x objective was taken. The image files were analysed on the Leica software. The software allows the merging

of multiple images and automatically performs a count on the total number of cells present on each slide.

2.8 Parsortix™

The Parsortix™ (Angle) separates CTCs from the blood based on their difference in size and compressibility compared to white blood cells. Details of the process are described in section 3.3.1.

2.8.1 Collection of samples

Blood samples were collected in a 10 ml CellSave™ tube as per the manufactures instructions.

2.8.2 Processing sample

The machine consists of a disposable cassette which filters the blood and captures the CTCs within the cassette. Figure 2.7.2.1 illustrates key components of the machine (141).

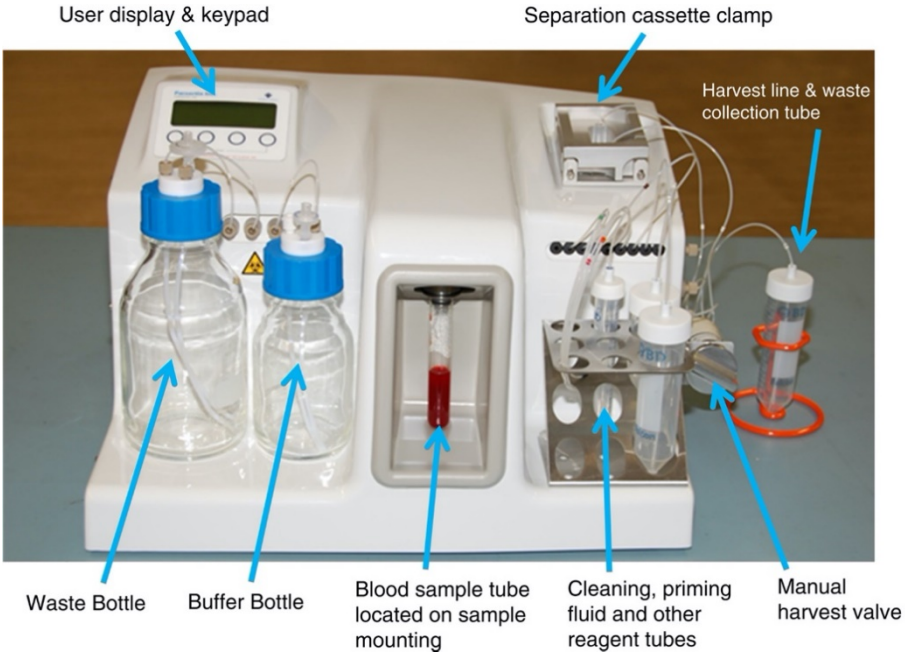


Figure 2.8.2.1: Parsortix™ PR1 Cell separation system

Components of the Parsortix™ cell separation system which consists of the blood sample tube mount, separation cassette holder, harvest line and waste collection tube and the buffer solution for cleaning and priming the machine.

Figure taken from (130)

A sample cassette was placed in the clamp and was flushed with PBS which was used as the buffer, this process takes approximately 60-90 minutes. The Parsortix™ has individual programmes for each step. The machine automatically determines the length of each programme. A new cassette was placed into the holder as shown in Figure 2.7.2.1. The first programme PX2_P was selected. This programme primes the new cassette with the buffer. The second programme was PX2_S99F. A 10 ml CellSave™ tube or 10 ml EDTA tube containing the blood sample at room temperature was loaded into the machine following the prompt. The blood was drawn into the machine and circulated into the cassette. This process took between 2-3 hours dependent upon the volume of blood processed. The waste was discarded into a separate bottle. Following completion of the second programme, the cells captured in the cassette can either be labelled within the cassette or be harvested into a separate container. Programme PX2_H was selected to harvest the cells. A 1.5 ml Eppendorf tube was placed under the harvest line and secured. The manual harvest valve was turned into the anti-clockwise position and cassette was flushed with 200 μl of PBS. The cell suspension is collected in the Eppendorf tube. The line was flushed with a further 1 ml of PBS to maximise cell recovery. The cells suspension was ready for downstream analysis. The machine was covered in foil and protected from light when cells conjugated with fluorescence antibodies were separated.

The machine was cleaned using the programme PTXC_2 in preparation for the next sample or user.

Chapter 3: Development and optimization of the protocol for isolating disseminated tumour cells from the blood and bone marrow

3.1 Introduction

The chapter focuses on the development and optimisation of the protocols for the isolation of circulating tumour cells from the blood and disseminated tumour cells from the bone marrow.

A protocol for the isolation of circulating tumour cells from peripheral blood with red cell lysis and immunomagnetic separation had been previously published(131). The enrichment and recovery using this method was reported at 50%. When the protocol was trialled with oesophageal cancer cell lines added to blood samples obtained from healthy volunteers, the enrichment and recovery of the oesophageal cancer cells was poor. The recovery was approximately 10-15%. In addition, the time for processing individual blood samples was over three hours. The focus on the initial experiments was to identify areas within this protocol which could be optimised to improve the enrichment and recovery of circulating tumour cells in the blood. The main areas of interest were the following: Collection and storage of the blood samples, red cell lysis using the BD Phosflow^{TM} Lyse/Fix buffer and reducing the time period for processing the blood sample

Bone marrow consists of cellular and non cellular components and present in the spongy part of the bone beneath the cortex. The main sites of bone marrow are the ribs, vertebrae, sternum and bones of the pelvis. It consists of stromal tissues, adipose tissue and haematopoietic progenitor cells. The haematopoietic progenitor cells develop into three main types of cells: white blood cells, red blood cells and platelets. Previous studies have demonstrated the successful isolation of disseminated tumour cells in the bone marrow from the rib in patients with oesophageal cancer using Ficoll-Hypaque density centrifugation ((112). This method relies upon the difference in size between the disseminated tumour cells and the other mononuclear cell within the bone marrow. The reported rates of detection using this method varied from 18-88%. The aim was to compare different methods and develop a protocol to enrich and isolate disseminated tumour cells from the bone marrow.

Another focus on this chapter is the evaluation of other methods used to isolate circulating tumour cells from the blood. Two methods were compared. Parsortix™ separates CTCs directly from blood based on their increased size in comparison to other monoculear cells within the blood. Easy Sep™ Direct human CTC enrichment which uses immunomagnetic beads to isolate CTCs from blood directly without the addition of fixative agents and with the potential to isolate CTCs directly into culture.

3.2 Validation and optimisation of the protocol for enumerating circulating tumour cells in peripheral blood

3.2.1 Optimisation of the red cell lysis in the protocol for enumerating circulating tumour cells in peripheral blood

Several experiments were designed to improve the CTC recovery rate and minimize the time for processing. Initial experiment was to evaluate the maximum volume of blood that could be processed per fixed volume of the BD Phosflow™ Lyse/Fix buffer. Four different ratios of blood : BD BD Phosflow™ Lyse/Fix buffer (1:5, 1:10, 1:20, 1:30) were tested for a fixed volume of 1 ml of blood per sample. Cells were counted following the red cell lysis and centrifugation stage. The recovery of white blood cells was calculated and the results illustrated in figure 3.2.1.1.

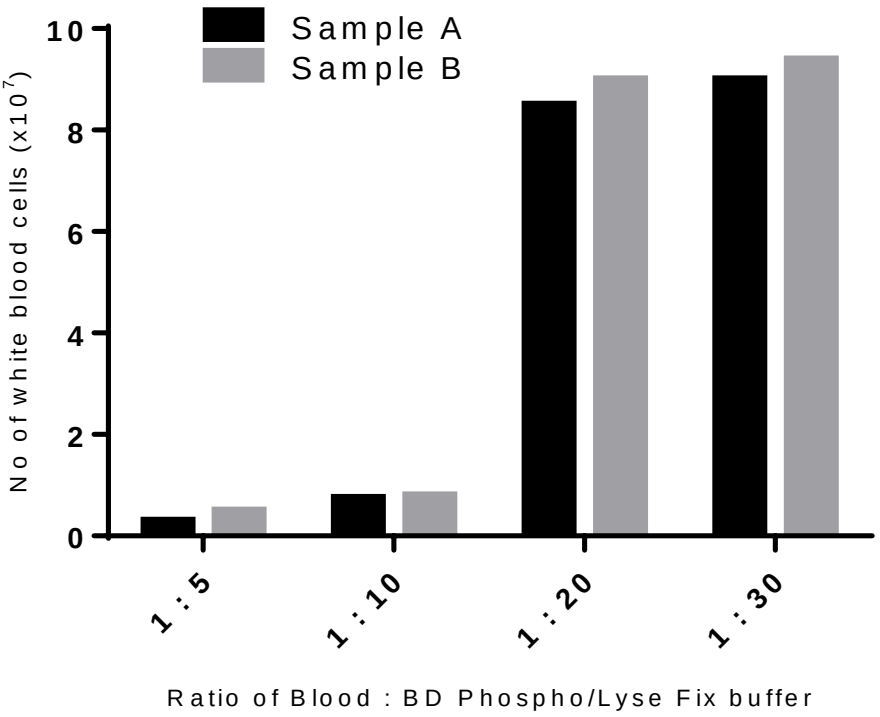


Figure 3.2.1.1: Mononuclear cell recovery with different ratios of blood : BD Phosflow™ Lyse/Fix buffer

Four pre-blocked (3% BSA) 50 ml Falcon tubes each with 1 ml of healthy volunteer blood were prepared. Diluted BD Phosflow Lys/Fix buffer pre-warmed at 37^{o}C was added to the blood at the following ratios to each individual tube, 1:5, 1:10, 1:20 and 1: 30. Each sample was incubated at 37^{0}C for 15 minutes. Then centrifuged at 500 g for 10 minutes. The supernatant was discarded and the cells in the pellet were counted with a haemacytometer. The experiment was repeated with another healthy volunteer sample. The approximated number of cells in the blood sample prior to red cell lysis was 4 x 10^7 in Sample A and 5 x 10^7 in Sample B.

The optimal ratio of blood : BD phosphor/lyse fix buffer was 1:20. A lower ratio led to a significant loss of the cellular component of the blood. Although a higher ratio led to an increase in the number of white blood cells recovered, the large volume of buffer required was not justifiable.

3.2.2 Comparing the collection and storage of peripheral blood sample with BD Vacutainer tube (EDTA) and CellSave™

The collection and storage of the peripheral blood was previously carried out using the CellSave™ tube which contains 300 µL of Na_2EDTA for clotting prevention and a cell surface preservative to maintain the morphology and cell surface antigen expression for phenotyping (ref manufacturer). The manufacturer specification stated that cells can be preserved in the CellSave™ tube for up to 96 hours at room temperature. BD Vacutainer™ tube contains EDTA and preserve cells up to 4 hours at room temperature. Initial experiments evaluating recovery of oesophageal cancer cell lines added to volunteer blood samples with the CellSave™ tube was poor, less than 15% of the mononuclear cells were recovered post red cell lysis. In addition, majority of the cells recovered were not healthy.

An experiment was designed to evaluate the recovery of the mononuclear cellular component from peripheral blood using the two different methods of collection and storage. Twelve ml of blood was obtained from a healthy volunteer, 4 ml was collected in a BD vacutainer EDTA tube and the other 8 ml of blood was collected and divided equally into four CellSave™ tubes. The samples were stored at room temperature. At two and four hours post collection, the blood sample in the BD vacutainer tube underwent red cell lysis with the BD Phosflow™ Lyse/Fix buffer. Post red cell lysis, a haemocytometer was used to perform a cell count and evaluate the enrichment of the mononuclear samples from the sample. Blood samples in the CellSave™ tubes underwent red cell lysis with the Phosflow™ Lyse/Fix buffer at the following intervals: 2, 12, 24 and 48 hours. Figure 3.2.2.1 illustrates the difference in the enrichment of the mononuclear component of the blood between the BD Vacutainer EDTA tube and CellSave™ tubes.

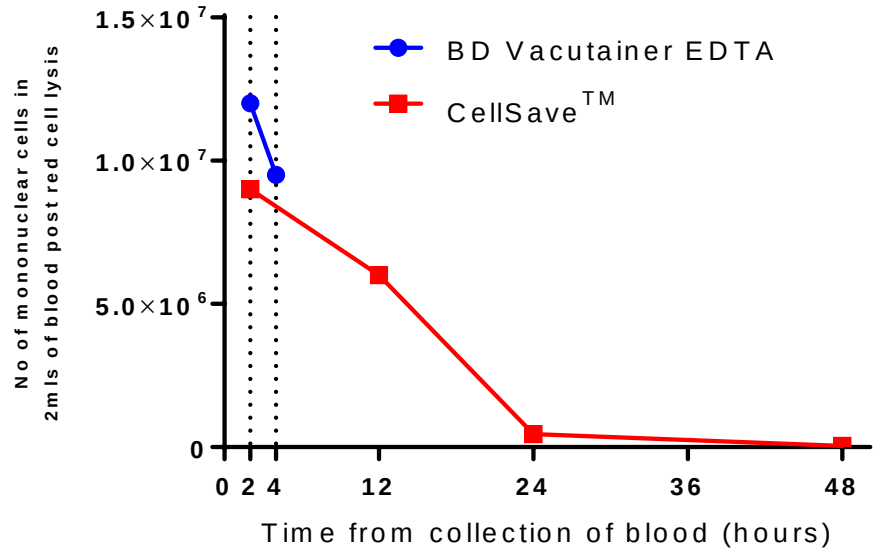


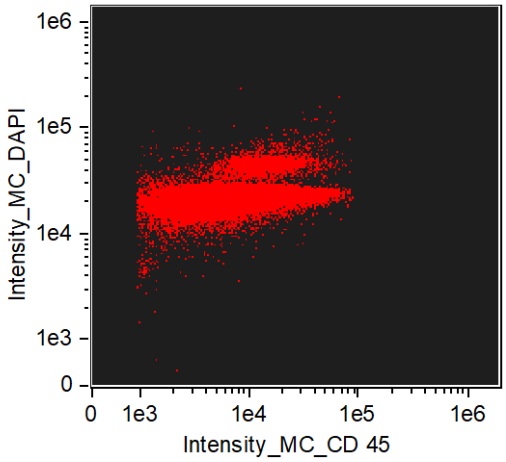
Figure 3.2.2.1: Comparison of the mononuclear cell recovery with two different methods of peripheral blood collection and storage

Two ml of blood collected and stored in BD Vacutainer EDTA tube for two hours was added to a pre-blocked (3% BSA) 50 ml Falcon tubes. Diluted BD Phosflow Lys/Fix buffer pre-warmed at 37°C was added to the blood at the following ratio 1:20. The sample was incubated at 37^{0}C for 15 minutes. Then centrifuged at 500 g for 10 minutes. The supernatant was discarded and the cells in the pellet were counted with a haemocytometer.

The experiment was repeated with blood stored in the CellSave™ tubes at the following time intervals from collection: 2, 12, 24 and 48 hours.

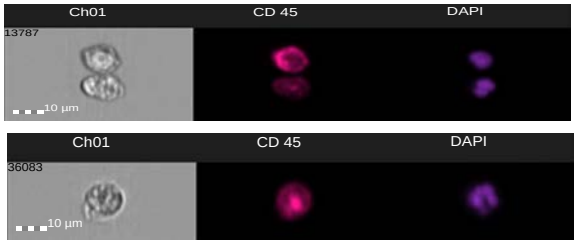
There was a significant drop in the number of viable mononuclear cells present in the sample collected and stored in the CellSave™ tube after 12 hours. As indicated in the Figure 3.2.2.1, at 24 hours following red cell lysis only a small fraction of the mononuclear cells were recovered and majority of the sample contained cellular debris. In addition, the enrichment of the mononuclear cellular component was superior with the blood samples collected and stored in the BD vacutainer EDTA tube at two hours when compared to the CellSave™ tube. Figure 3.2.2.2 illustrates the difference in the cell viability post red lysis between the two methods of storage.

CD 45 cells – 2 hours post storage BD

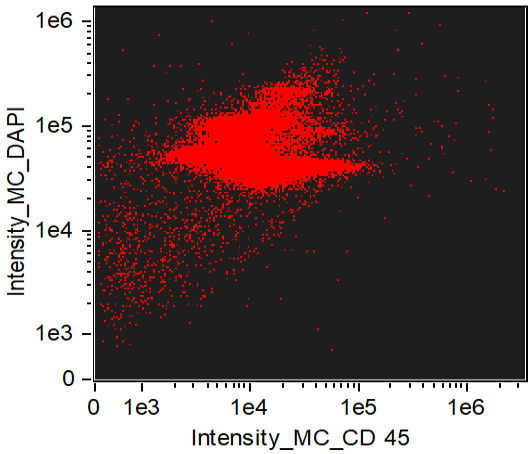


Intensity_MC_CD 45, Intensity_MC_DAPI

Population	Count	% Gated
CD 45 + & R9	89756	100



CD 45 cells – 2 hours post storage CellSave™ tube



Intensity_MC_CD 45, Intensity_MC_DAPI

Population	Count	% Gated
CD 45	61860	100

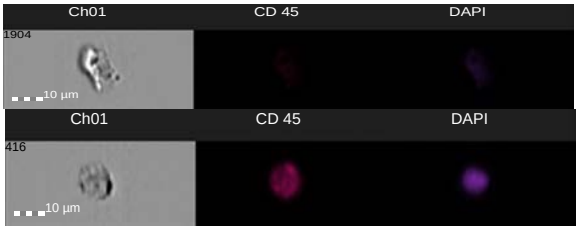


Figure 3.2.2.2: Comparison of the mononuclear cell recovery with two different methods of peripheral blood collection and storage on the high resolution imaging flow cytometer

Two milliliters of blood collected and stored in BD Vacutainer EDTA tube for two hours was added to a pre-blocked (3% BSA) 50 ml Falcon tubes. Diluted BD Phosflow Lys/Fix buffer pre-warmed at 37ºC was added to the blood at the following ratio 1:20. The sample was incubated at 37^{0}C for 15 minutes. Then centrifuged at 500 g for 10 minutes. The cell pellet was incubated with the following anti-bodies in 100 µl Permwash™ for 30 minutes: PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The cells were visualised on the high resolution imaging flow cytometer and analysis was performed on the IDEAS software.

The process was repeated with 2 ml of blood collected and stored in CellSave™ tube

Cells stored in the CellSave^{TM} tube had evidence of degradation and there was a higher amount of cellular debris as illustrated in Figure 3.2.2.2. This raises concerns regarding the viability of CTCs collected and stored in CellSave^{TM} tube when compared to the BD vacutainer EDTA tube.

3.2.3 Excluding the FcR blocking reagent from the protocol for enumerating circulating tumour cells in peripheral blood

The first step of the protocol involved incubating the blood with the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) for twenty minutes at 4^{o}C prior to red cell lysis. The primary function of the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) is to prevent non specific binding of anti-bodies to cells expressing the human Fc receptor, eg plasma B cells, monocytes and macrophages. It also increases the specificity of antibody labelling of rare target cells within the blood, eg circulating tumour cell, haematopoietic progenitor cells. The efficacy and function of the reagent was evaluated with the following experiment. Ten thousand OE19 cells cultured in RPMI were added to 1 ml of peripheral blood from a healthy volunteer. The volume of the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) required for the experiment was determined as per the manufacturer's datasheet. The sample was then incubated at 4^{o}C for 20 minutes with 80 µl FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec). The sample underwent red cell lysis. The cell pellet obtained post red cell lysis was incubated with the following antibodies for 30 minutes at room temperature: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (HER-2) antibody (Cat# 324414) at 1 in 100 dilution, Alexa Fluor 647 anti-human CD45 antibody (Cat # 30406) at 1 in 100 dilution and DAPI (10ug/ml) at 1 in 100 dilution. The sample was then analysed on the high resolution imaging flow cytometer. The experiment was repeated without the addition of the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec). Figure 3.2.3.1 illustrates the comparison between the two samples. The addition of the FcR reagent had no impact on the antibody binding. There was no evidence of non-specific binding of the anti-bodies in both samples as shown in Figure 3.2.3.2.

Sample A:

Sample with FcR block reagent

Sample B: Sample without FcR block

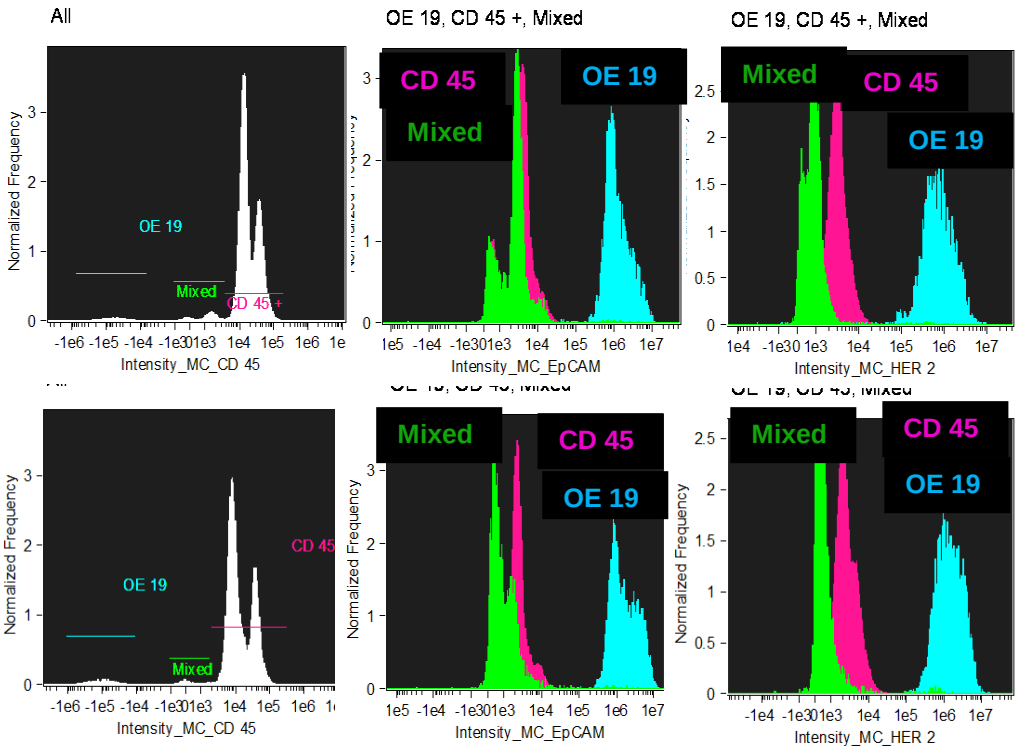


Figure 3.2.3.1: Comparison of the efficacy of the FcR block reagent

Ten thousand OE19 cells cultured in RPMI were trypsinised and added to 1 ml of blood. FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) 80 µl was added to the sample as per the standard protcol and incubated at 4^0C for 20 min. Red blood cells were lysed with BD phosphlo/lyse fix buffer. The cell suspension was incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (HER-2) antibody (Cat# 324414) at 1 in 100 dilution, Alexa Fluor 647 anti-human CD45 antibody (Cat # 30406) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. This was labelled Sample A.

The experiment was repeated without FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) and labelled Sample B. The cells were visualised on the high resolution image flow cytometer. Comparison of the data was performed with IDEAS software

Sample A: Sample with FcR block reagent

Sample B: Sample without FcR block reagent

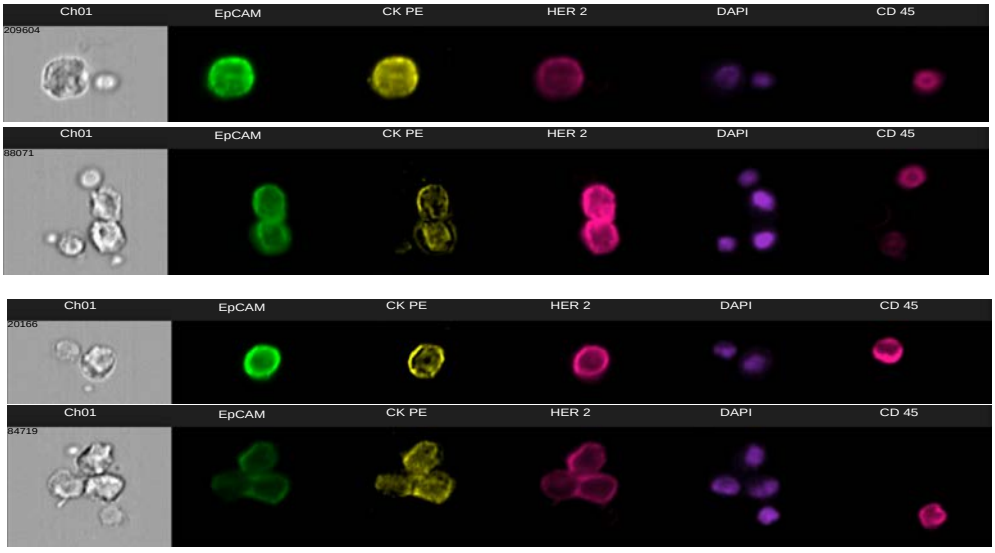


Figure 3.2.3.2: Images of OE 19 cells and CD45 positive cells from the high resolution imaging flow cytometer with and without the addition of the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec)

OE 19 cells express the epithelial markers: EpCAM, cytokeratin and human epidermal growth factor receptor (HER 2). In both samples there is clear differentiation between the white blood cells expressing the CD45 biomarker and the OE19 cells expressing the epithelial markers. There is no difference in the non specific antibody binding between the samples.

The addition of the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) increased the time taken for the protocol and the potential for a decrease in the recovery of the CTCs for no observable benefit as illustrated in the results. As a consequence, the FcR blocking reagent human (Cat no 135-059-901 Miltenyl Biotec) was excluded from the protocol.

3.3 Validation and optimisation of the protocol for enumerating disseminated tumour cells from the bone marrow

3.3.1 Identifying cells within control bone marrow samples

Prior to the density centrifugation, filtration of the bone marrow is required to exclude the stromal components. The size of the filter used in the studies for this purpose varied from 70 to 100 microns. The initial experiment focused on developing a protocol for enumerating disseminated tumour cells from the bone marrow and characterising the cellular population present in the bone marrow using the high resolution imaging flow cytometer.

Bone marrow from the femur was obtained from a patient undergoing an orthopaedic procedure. The marrow was excised from the section of bone and placed in 20 ml of sterile PBS. Two millilters of the solution was placed through a 70 micron filter and the filtrate was centrifuged at 500g for five minutes. The cell pellet post centrifugation was fixed in 4% paraformaldehyde for 10 minutes. The sample was centrifuged at 500g for 5 minutes. The supernatant was aspirated and discarded. The cell pellet was resuspended in 100 µl of PBS and incubated with the following primary and secondary antibodies listed in Section 2.3.5. The sample was analysed on the high resolution imaging flow cytometer as illustrated in Figure 3.3.1.1

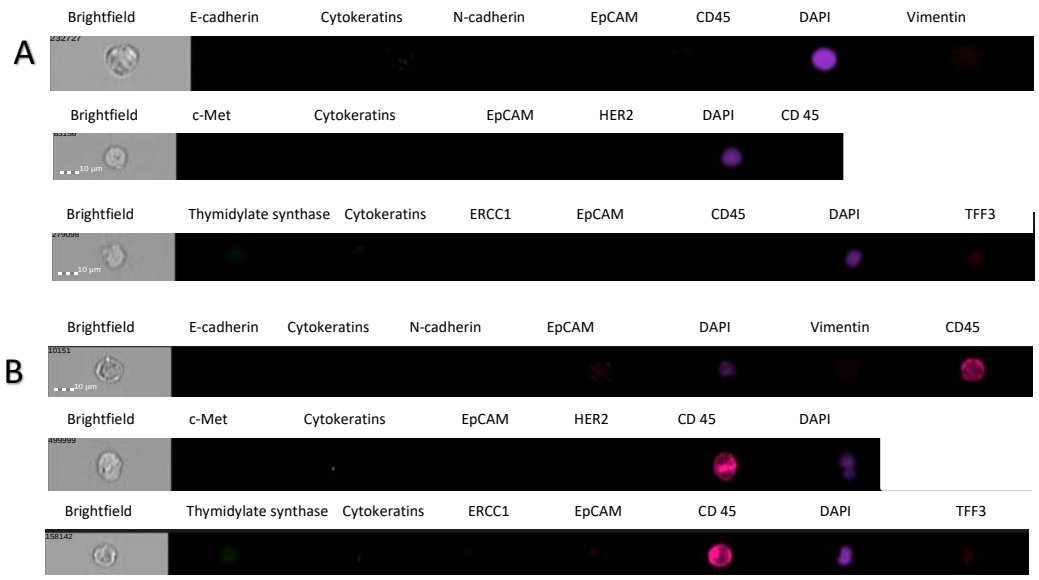
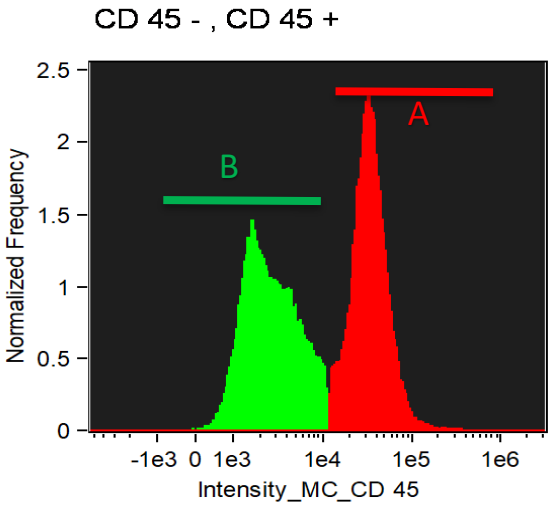


Figure 3.3.1.1: Images of cells present in the bone marrow of a healthy patient with the high resolution imaging flow cytometer

There were two distinct populations of cells identified based on the expression of the CD 45 biomarker. Population A consisted of cells that did not express any of the epithelial, mesenchymal and CD 45 biomarkers. These are likely the haematopoietic progenitor cells. Population B consisted of the white blood cells which expressed the CD 45 biomarker. There were no cells identified in the bone marrow which expressed either an epithelial and/or mesenchymal biomarker.



Intensity_MC_CD 45

Population	Count	% Gated
CD 45 - & R1	73342	100
CD 45 +	74005	100

Figure 3.3.1.2: Two population of cells within the control bone marrow sample

Population A consists of white blood cells and accounts for approximately 50% of the cellular population.

Population B consists of cells which did not express any of the epithelial, mesenchymal and CD 45 biomarkers. They account for the remainder of the population of cells within the bone marrow.

3.3.2 Development of a protocol for isolating disseminated tumour cells from the bone marrow

The next experiment focused on the isolation of the mononuclear component from the bone marrow. The remainder of the bone marrow solution approximately 18 ml post filtration was separated into four 20 ml Falcon tubes.

Two methods of enrichment were compared, density centrifugation using the Lymphoprep™ density gradient medium and red cell lysis with BD Phosflow™ Lyse/Fix buffer. Lymphoprep™ density gradient medium separates the components of the bone marrow into three distinct layer by centrifugation. The mononuclear component of the bone marrow is isolated from the plasma and red blood cells with a pipette. Four ml of the bone marrow was added to four ml of PBS containing 2% fetal bovine serum (FBS) into a 14 ml Falcon tube. Four ml of the Lymphoprep™ was carefully layered on top of the solution. The sample was centrifuged at 800g for 20 minutes at room temperature (15^{o}C-25^{o}C). The components of the bone marrow had separated into three layers: plasma, mononuclear cells and red cells. The mononuclear component was aspirated and placed in 5 ml of sterile PBS. A cell count was obtained using a haemacytometer. BD Phosflow™ Lyse/Fix buffer was used to lyse red cells and isolate the mononuclear component in four ml of the bone marrow solution using the standard protocol. Cell count post red cell lysis was obtained using a haemacytometer. Table 3.2.1 illustrates the efficacy of enrichment of the two methods.

Table 3.3.2.1 Comparison of enrichment of monocnuclear cells from the bone marrow using the Lymphoprep™ density gradient medium and BD Phosflow™ Lyse/Fix

	Lymphoprep™ density gradient	BD Phosflow™ Lyse/Fix
Sample A	1.8 x 10^5	2.5 x 10^6
Sample B	1.7 x 10^5	2 x 10^6

Enrichment of the mononuclear component of the bone marrow was higher with the BD Phosflow™ Lyse/Fix compared to the Lymphoprep™ density gradient. The next experiment focused on comparing the efficacy of enrichment and recovery of oesophageal cancer cell lines added to the bone marrow between the two methods. Twenty ml of bone marrow solution from

the femur of a patient undergoing an elective hip replacement was obtained. The sample was divided into four 20 ml falcon tubes containing 5 ml of bone marrow solution. SK-GT-4 cells cultured In RPMI were added at the following concentrations, 100 000 and 10000 cells in to the bone marrow prior to filtration. Figure 3.3.2.2 illustrates the difference in enrichment and recovery of SK-GT-4 cells added to the bone marrow between the two methods.

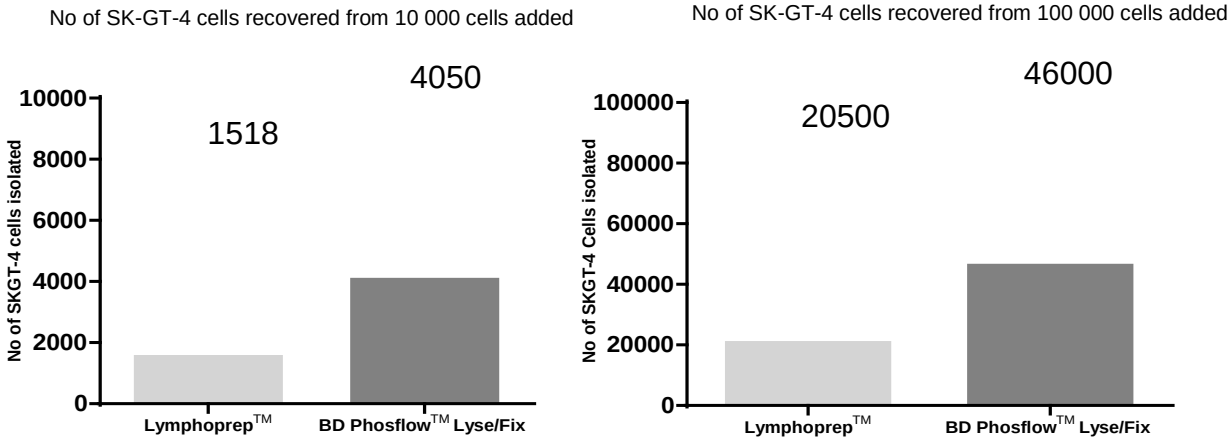


Figure 3.3.2.2 Comparison of enrichment and recovery of SK-GT-4 cells from the bone marrow using the Lymphoprep™ density gradient medium and BD Phosflow™ Lyse/Fix

One hundred thousand SK-GT-4 cells cultured in RPMI medium and fixed in 4% paraformaldehyde was added to five ml of bone marrow. The bone marrow was placed through a 70 micron filter. The filtrate was added to five ml of PBS containing 2% fetal bovine serum into a 20 ml Falcon tube. Five millitres of the Lymphoprep™ was carefully layered on top of the solution. The sample was centrifuged at 800g for 20 minutes at room temperature (15°C-25°C). The components of the bone marrow had separated into three layers: plasma, mononuclear cells and red cells. The mononuclear component was aspirated and centrifuged at 500g for 5 minutes. Immunomagnetic separation of the white blood cells was performed using EasySep™ Human CD45 Depletion (StemCell technologies). Post enrichment the cells were incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) (Cat 324214 BioLegend) at 1 in 100 dilution, DAPI (10 µg/ml) at 1in 100 dilution and PE/Cy7 anti-human CD45. The sample was imaged on the high resolution imaging flow cytometer and analysed using the IDEAS software. The experiment was repeated with 10 000 SK-GT-4 cells.

100 000 SK-GT-4 cells cultured in RPMI medium and fixed in 4% paraformaldehyde was added to five ml of bone marrow. The bone marrow was placed through a 70 micron filter. Red cells were lysed with BD Phosflow™ Lyse/Fix buffer. The cell pellet post lysis underwent immunomagnetic separation with EasySep™ Human CD45 Depletion (StemCell technologies) and labelling with the anti-bodies as described above. The experiment was repeated with 10 000 SK-GT-4 cells.

The recovery of the SK-GT-4 cells with the Lymphoprep™ density centrifugation medium was lower when compared to the BD Phosflow™ Lyse/Fix buffer, 15% and 20.5% compared to 46%

and 40%. The proportion of the SK-GT-4 cells recovered was dependant upon the number of cells present initially in the bone marrow solution, 46% and 20.5% recovery with 100 000 cells versus 40% and 15% recovery with 10 000 cells. The experiment was repeated with OE19 cells with 20mls of bone marrow obtained from the femur of a patient undergoing an elective orthopaedic hip replacement. Figure 3.3.2.3 illustrates the difference in enrichment and recovery of OE19 cells added to the bone marrow between the two methods.

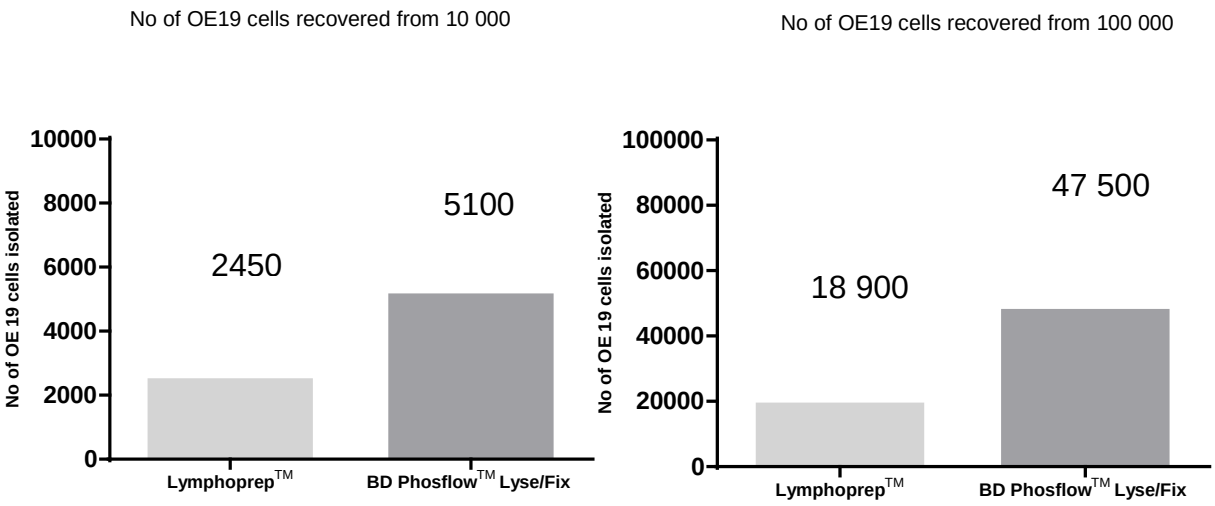


Figure 3.3.2.3 Comparison of enrichment and recovery of OE19 cells from the bone marrow using the Lymphoprep™ density gradient medium and BD Phosflow™ Lyse/Fix

One hundred thousand OE19 cells cultured in RPMI medium and fixed in 4% paraformaldehyde was added to 5 ml of bone marrow. The bone marrow was placed through a 70 micron filter. The filtrate was added to 5 ml of PBS containing 2% fetal bovine serum into a 20 ml Falcon tube. Five ml of the Lymphoprep™ was carefully layered on top of the solution. The sample was centrifuged at 800 g for 20 minutes at room temperature (15°C-25°C). The components of the bone marrow had separated into three layers: plasma, mononuclear cells and red cells. The mononuclear component was aspirated and centrifuged at 500 g for 5 minutes. Immunomagnetic separation of the white blood cells was performed using EasySep™ Human CD45 Depletion (StemCell technologies). Post enrichment the cells were incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) (Cat 324214 BioLegend) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The sample was imaged on the high resolution imaging flow cytometer and analysed using the IDEAS software. The experiment was repeated with 10 000 OE19 cells.

100 000 OE19 cells cultured in RPMI medium and fixed in 4% paraformaldehyde was added to 5 ml of bone marrow. The bone marrow was placed through a 70 micron filter. Red cells were lysed with BD Phosflow™ Lyse/Fix buffer. The cell pellet post lysis underwent immunomagnetic separation with EasySep™ Human CD45 Depletion (StemCell technologies) and labelling with the anti-bodies as described above. The experiment was repeated with 10 000 OE19 cells

Enrichment and recovery of the OE19 cells was lower with the Lymphoprep™ density gradient medium in comparison to the BD Phosflow™ Lyse/Fix buffer, 24.5% and 18.9% versus 51% and 47.5%. The difference in recovery in both experiment is attributed to a number of reasons. The successful isolation of the mononuclear cell component from the bone marrow using the Lymphoprep™ density gradient medium relies upon a number of factors. Accurate layering of the bone marrow sample onto the Lymphoprep™ density gradient medium prior to centrifugation is required. Contamination at this stage prevents the separation of the components of the bone marrow. In addition, the recovery of the mononuclear component of the bone marrow is reliant upon the presence of a defined layer of mononuclear cells post centrifugation. Furthermore, there is a potential to lose cells during the extraction of the cells from the Lymphoprep™ density gradient medium. Based on these experiments, the decision was made to use the BD Phosflow™ Lyse/Fix buffer to perform the red cell lysis and isolated the mononuclear cellular component of the bone marrow.

3.4 Other methods for isolating CTCs from blood

3.4.1 Parsortix™

There are a number of methods to isolate CTCs from whole blood as described previously. CTCs can be isolated based on their morphological characteristics, eg size, shape. CTCs are hypothesised to be larger than white blood cells, approximately > 8 μm in size. The Parsortix™ isolates CTCs from whole blood based on the difference in size and compressibility between CTCs and white blood cells. Whole blood stored in a EDTA tube is passed through a filtration cassette that consists of a network of capillaries. As the blood passes through the capillaries, cells that are > 10 um in size are trapped within the lumen and captured in the filtration cassette. There are two potential methods for identification and isolation of the CTCs post filtration. The cells captured in the cassette can be stained directly with up to four different fluorescent antibodies and visualised on a light microscope. The cells captured in the cassette can be collected directly into an Eppendorf for further analysis. The Parsortix™ has a number of potential benefits when compared to the CellSearch™ method. CTCs are isolated based on their size and not their biomarker expression. Thus CTCs undergoing EMT or which express mesenchymal markers are isolated. In addition, the time taken for the process is short when

compared to the immunomagentic depletion method, approximately 3 hours to process 10 ml of blood.

The following experiment compared the efficiency of recovery of SK-GT-4 cells added to blood between Parsortix™ and Easy Sep™ immunomagnetic depletion. SK-GT-4 cells cultured in RPMI medium and fixed in 4% paraformaldehyde were labelled with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate). Ten thousand SK-GT-4 cells were added to 5 ml of blood from a healthy human volunteer. The sample was placed in the Parsortix™. Cells post filtration were collected from the cassette into a 1.5ml Eppendorf. The cells were imaged on the high resolution imaging flowcytometer. A cell count was performed. The experiment was repeated with 5 000 SK-GT-4 cells. The experiment was repeated with the standard protocol for Easy Sep™ immunomagnetic depletion as described before. The cells were imaged on the high resolution imaging flowcytometer post enrichment. A cell count was performed. Figure 3.3.1.1 illustrates the difference in recovery of SK-GT-4 cells between the two methods.

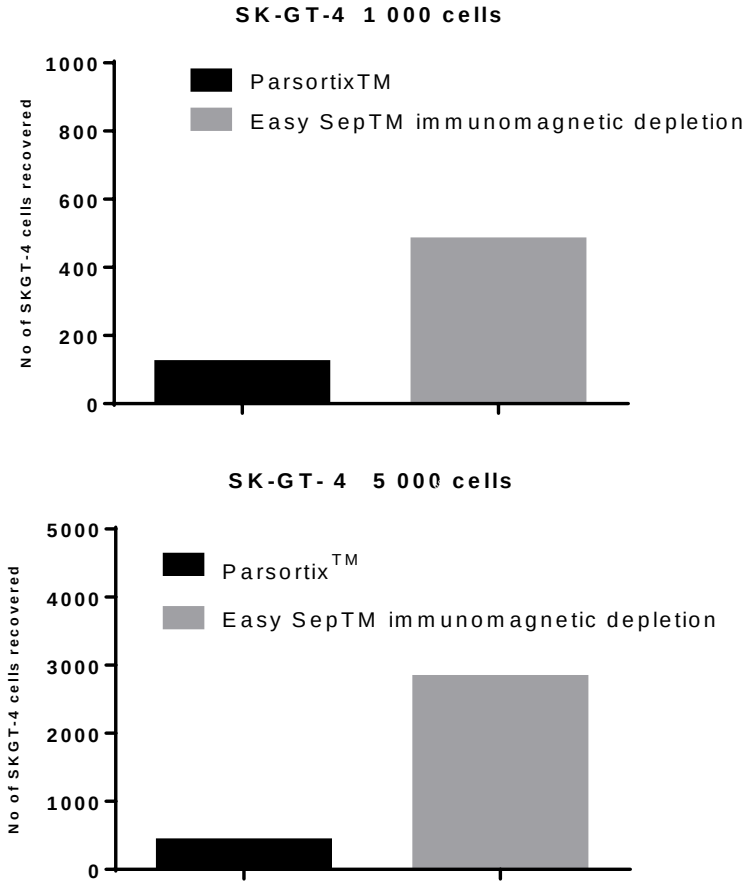
Figure 3.4.1.1: Comparison of enrichment and recovery of SK-GT-4 cells from blood between Parsortix™ and Easy Sep™ immunomagnetic depletion

No of SK-GT-4 cells added to 5mls of blood	**Easy Sep™ immunomagnetic depletion – No of SK-GT-4 cells recovered (%)**	**Parsortix™ – No of SK-GT-4 cells recovered (%)**
10 000	5 240 (54%)	3 158 (32%)
5 000	2 350 (47%)	1 350 (27%)

The number of SKT-4 cells recovered was less with the Parsortix™ when compared to the Easy Sep™ immunomagnetic depletion, 32% versus 52%. The lower rate of enrichment could be attributed to the additional step of recovering the cells from the filtration cassette with the Parsortix™ and imaging with the high resolution imaging flow cytometer rather than direct visualisation of the cells in the cassette with confocal microscopy. With clinical samples, positive identification of the CTCs is required with epithelial and/or mesenchymal biomarkers conjugated with fluorophores. This can be carried out by incubating the cells isolated within the cassette with antibodies conjugated with fluorophores. The CTCs are visualised directly in the

cassette using a confocal microscope. However only a limited range of fluorophores that can be imaged on the confocal microscope. Thus the number of biomarkers that can be used is limited. The other method of positive identification of CTCs, involves recovering the cells from the cassette post filtration into an Eppendorf. Labelling the cells with biomarkers which can be visualised on the high resolution imaging flow cytometer which has a wider spectrum of fluorophores that can be detected when compared to the confocal microscope.

The next experiment focused on labelling cells with antibodies post filtration in the Parsortix^{TM} and comparing it with Easy Sep^{TM} immunomagnetic depletion and subsequent imaging on the high resolution imaging flow cytometer. Ten thousand SK-GT-4 cells cultured in RPMI and fixed in 4% paraformaldehyde were added to 5 ml of blood. The sample was placed in the Parsortix^{TM}. The cells isolated in the cassette post filtration were collected in a 1.5ml Eppendorf and incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210), PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) and DAPI (10 µg/ml). The cells were then imaged on a high resolution imaging flow cytometer. The experiment was repeated with 5 000 and 1 000 SK-GT-4 cells. Figure 3.4.1.2 illustrates the difference in recovery of SK-GT-4 cells and OE19 cells between the two methods.



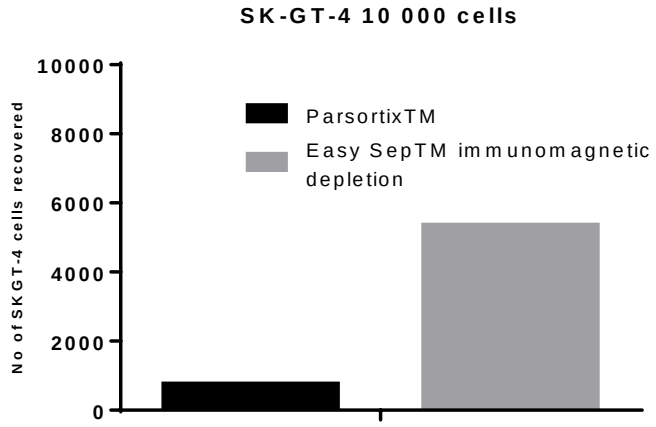


Figure 3.4.1.2: Comparison of enrichment and recovery of SK-GT-4 from blood between Parsortix™ and Easy Sep™ immunomagnetic depletion

Ten thousand SK-GT-4 cells were cultured in RPMI medium and fixed with 4% paraformaldehyde were added to 5 ml of blood from a healthy volunteer. The sample was placed in the Parsotix™. The cell suspension isolated post filtration was collected in a 1.5 ml Eppendorf and incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The SK-GT-4 cells were visualised and a count was performed on the high resolution imaging flow cytometer. The experiment was repeated with 5 000 and 1 000 SK-GT-4 cells.

The experiment was repeated with ten thousand SK-GT-4 cells cultured in RPMI medium fixed with 4% paraformaldehyde were added to 5 ml of blood from a healthy volunteer. Red blood cells were lysed with the BD Phosflow™ Lyse/Fix buffer. The cell pellet isolated post red cell lysis underwent Easy Sep™ immunomagnetic depletion. Post enrichment, the cells were labelled with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The SK-GT-4 cells were visualised and a count was performed on the high resolution imaging flow cytometer. The experiment was repeated with 5 000 and 1 000 SK-GT-4 cells.

The Parsortix™ had significantly lower rates of recovery when compared to the Easy Sep™ immunomagnetic depletion irrespective of the number of SK-GT-4 cells being present in the blood. Identification of biomarkers present in the nucleus and/or cytoplasm of cells requires an additional step of permeabilisation with saponin or Permwash™ to allow the antibodies to traverse the cell wall. This is an additional step in the process of labelling antibodies and would not be possible with the cells isolated in the casette with Parsortix™. The following experiment evaluated the difference in recovery with this additional step between Parsortix™ and Easy Sep™ immunomagnetic depletion. Ten thousand FLO1 cells were added to 1 ml of blood and placed in the Parsortix™. The cell suspension post isolation was labelled with the following antibodies: PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414), DAPI (10 µg/ml) and

Cytokeratin Monoclonal PE Antibody (Clone C-11)(CAY10478-1 ea). Figure 3.4.1.3 illustrates the difference in recovery between Parsortix™ and Easy Sep™ immunomagnetic depletion

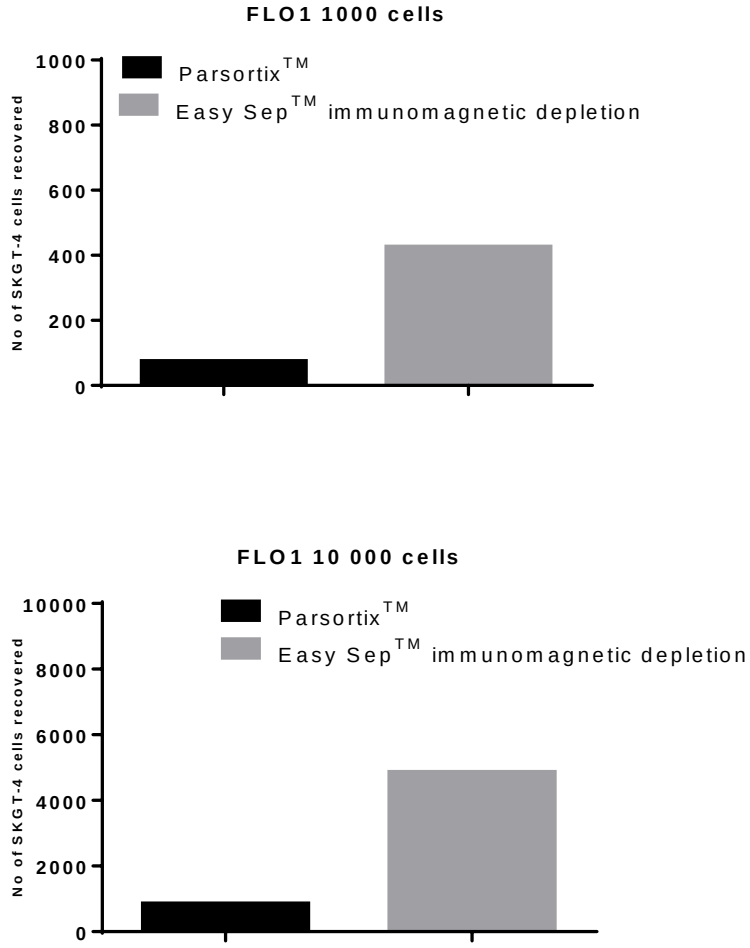


Figure 3.4.1.3: Comparison of enrichment and recovery of FLO1 cells from blood between Parsortix™ and Easy Sep™ immunomagnetic depletion

Ten thousand FLO1 cells were cultured in DMEM medium and fixed with 4% paraformaldehyde were added to 5 ml of blood from a healthy volunteer. The sample was placed in the Parsotix™. The cell suspension, approximately 120 µl was isolated post filtration was collected in a 1.5ml Eppendorf and was centrifuged at 500 g for 5 min. There was no visible cell pellet post centrifugation. 20 µl was removed and 100 µl of Permwash™ was added and incubated with the following antibody for 30 minutes: Cytokeratin Monoclonal PE Antibody (Clone C-11)(CAY10478-1 ea) at 1 in 20 dilution. After 30 minutes, the cell suspension was centrifuged at 500 g for 5 min and 100 µl was discarded. The cell suspension was then incubated with the following antibodies: PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The FLO1 cells were visualised and a count was performed on the high resolution imaging flow cytometer. The experiment was repeated with 5 000 FLO1 cells.

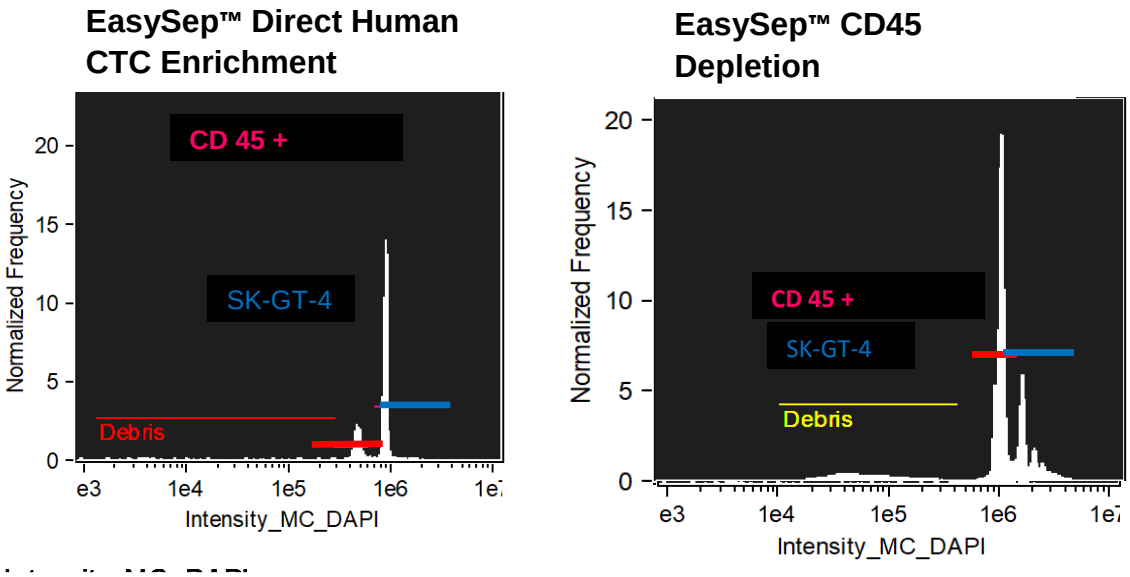
The experiment was repeated with the Easy Sep™ immunomagnetic depletion. Labelling of the cell suspension post enrichment was carried out as per the standard protocol.

Both experiments highlighted a number of issues with the Parsortix™. Recovery of cells pre-labelled with the CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) was lower with the Parsortix™. Furthermore, labelling cells isolated within the cassette was challenging. Only a small number of fluorophores could be employed to identify cells using the confocal microscope. The secondary option was to recover the cells from the cassette directly into a 1.5 ml Eppendorf. The manufacturer commented that approximately 20% of cells were lost during this transfer. This contributes to the overall lower recovery when compared to Easy Sep™ immunomagnetic depletion. In addition, there was no visible cell pellet in the Eppendorf post isolation and thus downstream labelling of antibodies with the standard protocol proved challenging. Overall, Parsortix™ proved to be less successful in practice than predicted.

3.4.2 EasySep™ Direct Human CTC Enrichment

EasySep™ Direct Human CTC Enrichment enriches CTCs directly from whole blood by negative selection using immunomagentice beads. Haematopoietic cells and platelets within the blood are separated from CTCs with antibodies recognizing CD2, CD14, CD16, CD19, CD45, CD61, CD66b, and Glycophorin A surface markers. The antibodies are combined with EasySep™ Direct RapidSpheres™, and separated using an EasySep™ magnet. This method contains no fixative agent and thus CTCs isolated can be placed directly into culture. The following experiements evaluated the efficacy of this method to isolate CTCs from blood.

The first experiment compared the recovery of SK-GT-4 cells with EasySep™ Direct Human CTC Enrichment and Easy Sep™ immunomagnetic depletion. Three hundred thousand SK-GT-4 cells were added to 2 ml of blood from a healthy volunteer. The sample underwent immunomagnetic separation with the EasySep™ Direct Human CTC Enrichment as per the standard protocol. Post enrichment, the cells were labelled with the following antibodies: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210), PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) and DAPI (10µg/ml). The cells were visualised on the high resolution imaging flow cytometer. The experiment was repeated with Easy Sep™ immunomagnetic depletion. Figure 3.4.2.1 compares the recovery of SK-GT-4 cells with the two methods.



Population	Total No of objects
SK-GT-4	1261 (25%)
CD45+	3502 (68%)
Debris	360 (7%)

Population	Total No of Objects
SK-GT-4	42 605 (43%)
CD45+	44095 (45%)
Debris	12 738 (13%)

Figure 3.4.2.1: Comparison of enrichment and recovery of SK-GT-4 cells from blood between EasySep™ Direct Human CTC Enrichment and Easy Sep™ immunomagnetic depletion

Three hundred thousand SK-GT-4 cells cultured in RPMI media and fixed with 4% paraformaldehyde were added to 2 ml of blood from a healthy volunteer and transferred to a 5 mL (12 x 75 mm) polystyrene round-bottom tube. The sample was incubated with 100 µl of EasySep™ Direct Human CTC Enrichment solution for 5 minutes at room temperature. After which, 100 µl EasySep™ Direct RapidSpheres™ was added and 2mls of 3% BSA was added to the sample. The sample was incubated for 10 minutes at room temperature and then placed in a EasySep™ magnet for 5 minutes. After 5 minutes, in one continuous movement, the magent and the tube is picked up and inverted and the the enriched cell suspension is transferred into another 5 ml tube. The process was repeated with another 100 µl EasySep™ Direct RapidSpheres™ added to enriched cell suspension. The cell suspension post immunomagentic depletion was incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (CD 45) antibody (Cat# 324414) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The SK-GT-4 cells were visualised and a count was performed on the high resolution imaging flow cytometer. The experiment was repeated with the standard protocol for Easy Sep™ immunomagnetic depletion.

The graphs illustrate the proportion of SK-GT-4 cells recovered with the two method. The experiment was repeated with the Easy Sep™ immunomagnetic depletion. Labelling of the cell suspension post enrichment was carried out as per the standard protocol.

The Easy Sep™ immunomagnetic depletion was superior in terms of recovery of the SK-GT-4 cells, 43% of cells recovered versus 25% with the EasySep™ Direct Human CTC Enrichment. As illustrated, there was a large amount of debris present in the cell suspension with the EasySep™ Direct Human CTC Enrichment method. The debris conisted of a complex of the EasySep™ Direct RapidSpheres™ combined white blood cells. This combined with the smaller number cells being present post depletion contributed to the lower recovery of SK-GT-4 cells with the EasySep™ Direct Human CTC Enrichment. Furthermore, EasySep™ Direct Human CTC Enrichment was not superior in terms of CD 45 depetion. Contanimation of white blood cells was present in both samples.

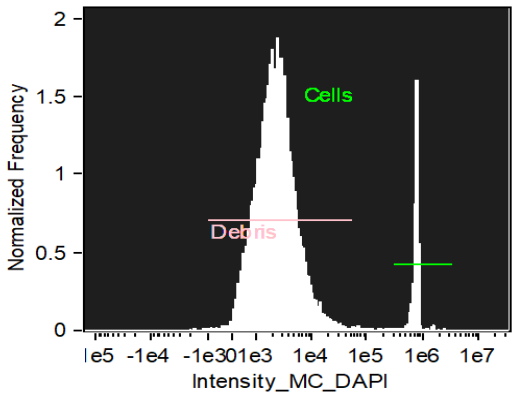
The second experiment focused on the efficiency of recovery of OE19 cells from blood between the two methods. Ten thousand OE19 cells cultured in RPMI media were added to two ml of blood from a healthy volunteer and transferred to a 5 mL (12 x 75 mm) polystyrene round-bottom tube. The sample underwent enrichment with the EasySep™ Direct Human CTC protocol as described before**.** The cell suspension post immunomagentic depletion was incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 anti-human CD340 (erbB2/HER-2) Antibody (Cat #324414) at 1 in 100 dilution, CD45 Alexa Fluor® 647-conjugated (Cat #304018) at 1 in 100 dilution and DAPI (10µg/ml) at 1 in 100 dilution. The OE 19 cells were visualised and a count was performed on the high resolution imaging flow cytometer. The experiment was repeated with 5 000 and 1 000 OE 19 cells. The experiment was repeated with the standard protocol for Easy Sep™ immunomagnetic depletion. Table 3.4.2.2 compares the recovery of OE19 cells between the two methods. Figure 3.4.2.3 illustrates the difference in recovery of 1 000 OE19 cells as visualised on the high resolution imaging flow cytometer.

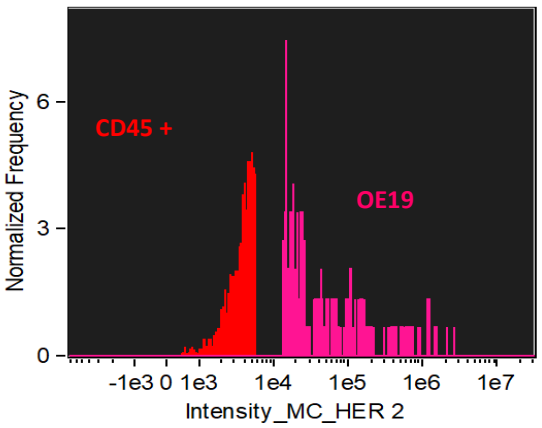
Table 3.4.2.2: Comparison of enrichment and recovery of OE 19 cells from blood between EasySep™ Direct Human CTC Enrichment and Easy Sep™ immunomagnetic depletion

No of OE19 cells added to 2 mls of blood	**Easy Sep™ immunomagnetic depletion – No of OE19 cells recovered (%)**	**EasySep™ Direct Human CTC Enrichment – No of OE19 cells recovered (%)**
10 000	Sample lost	980 (9.8%)

5 000	3 400 (34%)	220 (2.2%)
1 000	503 (50%)	148 (15%)

EasySep™ Direct Human CTC Enrichment - 1000 OE19 cells

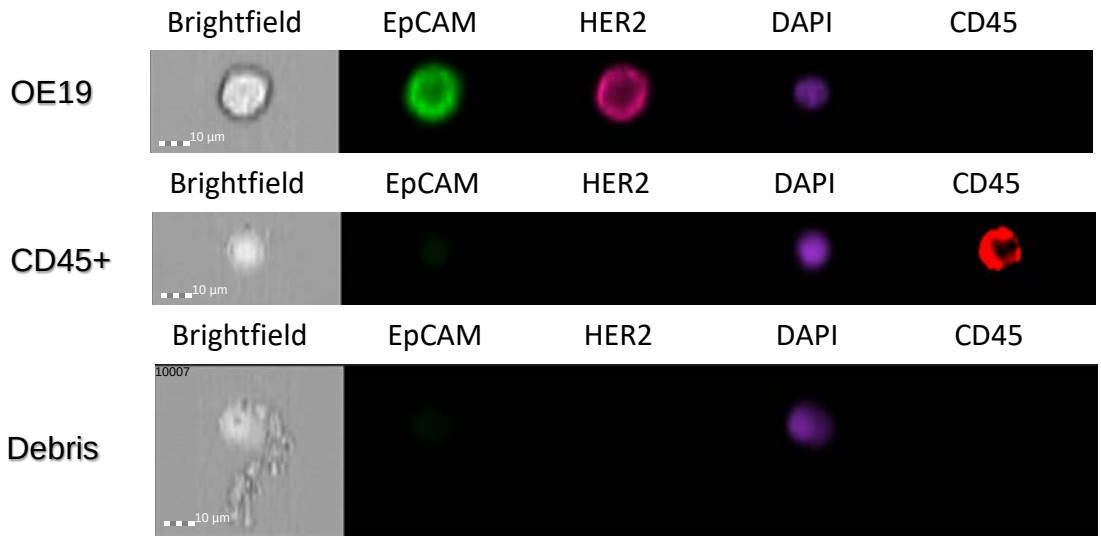




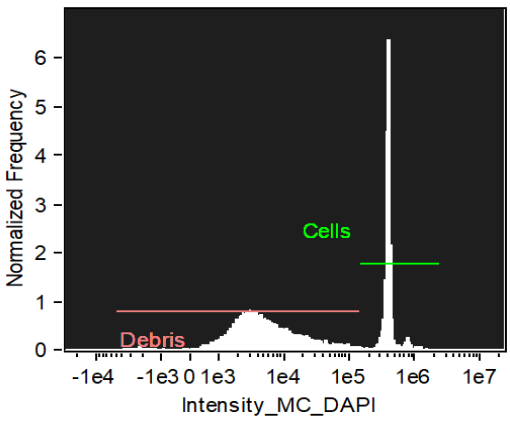
Population	Total No of objects (28 640)
Cells	2138 (7%)
Debris	26 194 (92%)

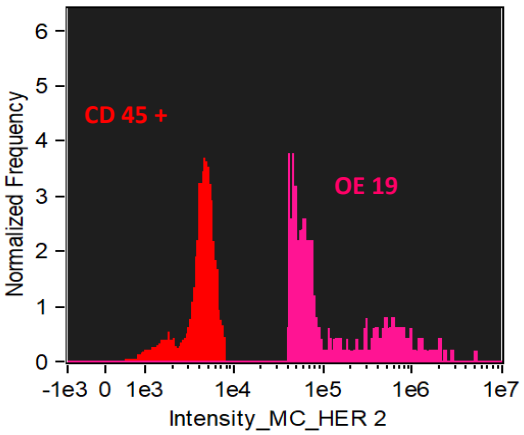
Population	Total No of Cells (2138)
OE19	148 (7%)
CD45+	1990 (93%)

Images of cells captured on the high resolution imaging flow cytometer post EasySep™ Direct Human CTC Enrichment



EasySep™ CD45 Depletion - 1000 OE19 cells





Population	Total No of objects (35 169)
Cells	13 074 (37%)
Debris	21 975 (63%)

Population	Total No of Cells (13 074)
OE19	503 (4%)
CD45+	12 571 (96%)

Images of cells captured on the high resolution imaging flow cytometer post EasySep™ CD45 Depletion

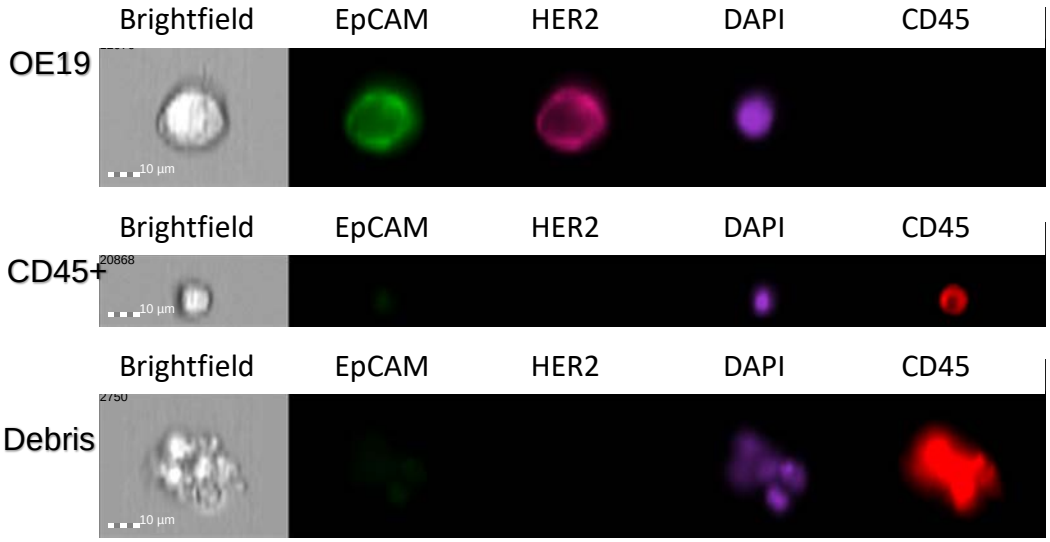


Figure 3.4.2.3: Comparison of enrichment and recovery of OE 19 cells from blood between EasySep™ Direct Human CTC Enrichment and Easy Sep™ immunomagnetic depletion and Images of the cells captured on the high resolution imaging flow cytometer

One thousand OE19 cells cultured in RPMI media were added to two ml of blood from a healthy volunteer and transferred to a 5 mL (12 x 75 mm) polystyrene round-bottom tube. The sample underwent enrichment with the EasySep™ Direct Human CTC protocol as described before**.** The cell suspension post immunomagentic depletion was incubated with the following antibodies for 30 minutes: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 anti-human CD340 (erbB2/HER-2) Antibody (Cat #324414) at 1 in 100 dilution, CD45 Alexa Fluor® 647-conjugated (Cat #304018) at 1 in 100 dilution and DAPI (10µg/ml) at 1 in 100 dilution. The OE 19 cells were visualised and a count was performed on the high resolution imaging flow cytometer. The experiment was repeated with the standard protocol for Easy Sep™ immunomagnetic depletion.

The recovery of OE19 cells was lower with the EasySep™ Direct Human CTC Enrichment. As illustrated in Figure 3.4.2.3, only 15% of the 1 000 OE19 cells were recovered. In addition, the amount of contamination of the sample with white blood cells was similar. As mentioned, before there was a large amount of cellular debris with the EasySep™ Direct Human CTC Enrichment method. This contributes to the lower rates of recovery when compared to the Easy Sep™ immunomagnetic depletion.

The next experiment focused on the evaluating the viability of cells for culture post isolation with the EasySep™ Direct Human CTC Enrichment. One hundred thousand SK-GT-4 cells cultured in RPMI medim were added to 2 ml of blood from a healthy human volunteer. The sample underwent EasySep™ Direct Human CTC Enrichment under sterile conditions. A cell count was carried out on the cell suspension using a haemocytomter. The cell suspension post enrichment was placed directly into a 75cm^3 **Corning®** flask containing 20 ml of RPMI with 20% FCS. Cells within the flask were imaged at this point and at the following intervals: 24, 48 and 72 hours. The experiment was repeated with one hundred thousand FLO1 cells. The cell suspsension post enrichment was placed in a 75cm^3 **Corning®** flask containing 20 ml of DMEM with 20% FCS. Figure 3.4.2.4 illustrates the images of the cells obtained with light microscopy at the following time intervals.

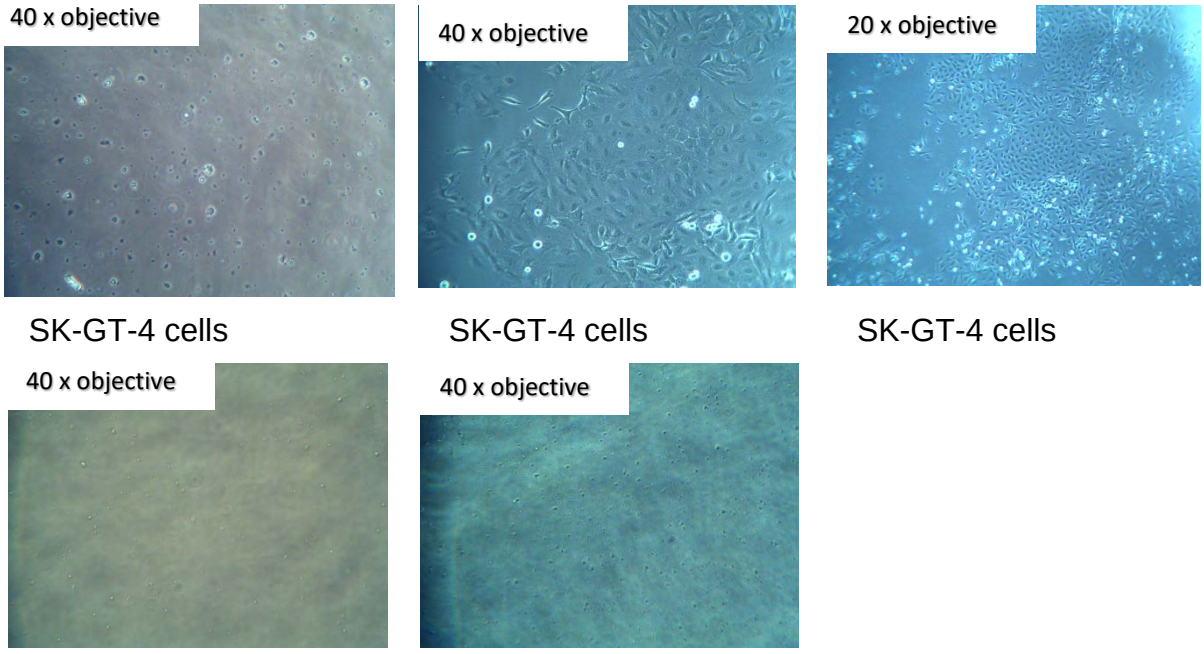


Figure 3.4.2.4: Images of SK-GT-4 and FLO 1 cells in culture following isolation with EasySep™ Direct Human CTC Enrichment

One hundred thousand SK-GT-4 cells were added to 2 ml of blood. The cell suspension post enrichment was placed directly into a 75cm^3 **Corning®** flask in 20 ml of RPMI with 20% FCS. Cells within the flask were imaged at this point and at the following intervals: 24, 48 and 72 hours. The experiment was repeated with one hundred thousand FLO1 cells. The cell suspsension post enrichment was placed in a 75cm^3 **Corning®** flask in 20 ml of DMEM with 20% FCS.

There were 15 000 cells isolated post enrichment with the SK-GT-4 sample. There were viable cells present at Day 2. The cells at this stage had become adherent to flask and were dividing. Although there were 3000 cells isolated post enrichment with the FLO1 sample, there were no viable cells present at Day 3 in culture. As noted in Figure 3.3.2.4, there was a large amount of debris present in the sample. Results from this experiment illusrate that the cells post EasySep™ Direct Human CTC Enrichment are viable and able to grown in culture. However, as described with earlier experiments, recovery of cells with EasySep™ Direct Human CTC Enrichment is poor. Less than 15% of cells are recovered from the blood. Thus a large of number of CTCs need to be present in the blood sample for successful isolation and culture with this method.

3.5 Discussion

One of the aims of the chapter was to address the concerns regarding the lower recovery rates of oesophageal cancer cell lines from peripheral blood with the original protocol. The recovery rate varied between 20-25%. The most important modification of the protocol was the change in the collection and storage method used when processing the peripheral blood sample. Collection of the blood sample using the BD vacutainer EDTA tube and processing within four hours has shown to significantly improve the recovery of the cells and preserve their viability. Amendments to the protocol, primarily omission of the FcR blocking agent led to a reduction in the processing cost per sample and reduced the time for processing. Other changes during the protocol revision was changes to the anti-body labelling. Overall the changes to the protocol reduced the time taken for processing a sample in preparation for analysis on the high resolution imaging flow cytometer from six hours to 4 hours.

The development of the protocol for isolating disseminated tumour cells from bone marrow focused on adapting previous methods described in literature. However, the recovery rates using the Lymphoprep™ density gradient medium was poor. Adapting the protocol used for isolating CTCs from peripheral blood proved more successful in achieving higher rates of recovery whilst preserving the viability of the cells.

As described in the literature there are various methods for isolating CTCs from blood. The primary focus of this chapter was to evaluate methods to isolate CTCs directly from blood into culture. The Parsortix™ was promising in the initial concept of isolating CTCs directly from peripheral blood into a filtration cassette. However, further analysis of the CTCs post isolation proved challenging. Labelling the isolated cells with anti-bodies was difficult and as a consequence the recovery of CTCs was significantly lower when compared to the Easy Sep™ immunomagnetic depletion protocol. The EasySep™ Direct Human CTC Enrichment used a two stage immunomagnetic separation to isolate CTCs from blood. The processing time was less than the current protocol using Easy Sep™ immunomagnetic depletion. However, recovery of oesophageal cancer cell lines from peripheral blood was lower with EasySep™ Direct Human CTC Enrichment when compated to Easy Sep™ immunomagnetic depletion. The EasySep™ Direct Human CTC Enrichment was successful in isolating oesophgeal cancer cell lines directly into culture. This method maintains the viability of the cells as illustrated by the presence of

growth of the cell lines up to 48 hours post isolation from blood. Although the experiment was promising, the low rates of recovery means that successful culture require a high number of CTCs being present per ml of peripheral blood. Further evauation is required to assess the efficacy of this method in isolating smaller number of oesophageal cancer cells from the blood. In addition, the method could be adapted to culture disseminated tumour cells from the bone marrow.

Chapter 4: Single cell Sorting

4.1: Introduction

The role and function of CTCs in the development and progression of cancer remains unknown. CTCs are a heterogeneous population of cells. The origins of the sub-populations remains poorly understood. It is thought that CTCs are derived from the primary tumour and express epithelial markers (142, 143). Previous studies have relied upon isolation of CTCs based on the expression of epithelial markers (128-132). However, a proportion of CTCs undergoing EMT express mesenchymal markers and are not detected (98). In addition, studies have shown that molecular expression and phenotype of CTCs can be different to that of the primary tumour (144-146) Isolation of individual circulating tumour cells is a challenge due to the low number being present in blood (65, 147). Developing a method which isolates CTCs using a wide array of biomarkers is vital in identifying the molecular origin of the cells and characterising the heterogeneity.

The primary aim of the work described in this chapter was to develop a protocol for isolation of individual circulating tumour cells based on expression of epithelial biomarkers from blood of patients with oesophageal adenocarcinoma. The intention is to isolate singles cells from different populations of CTCs based on the expression of specific biomarkers that could be sequenced using NextSeq 500 v2 sequencing and bioinformatics analyses. An additional aim was to develop a protocol for culturing circulating tumour cells from blood of patients with oesophageal adenocarcinoma.

The first stage of the method for red cell lysis was derived from the protocol described in chapter 2 for enrichment and isolation of circulating tumour cells from blood. The mononuclear cellular component isolated following the lysis, is labelled with fluorophores conjugated to anti-human CD45 antibody, epithelial biomarkers and DAPI. The cell pellet is suspended in 2 ml PBS and placed in a 6 ml FACS tube prior to analysis on the flow cytometer. The BD FACSAria™ Fusion is a high speed flow cytometer that allows single cells to be isolated. The machine has five lasers at wavelengths 355 nm, 405 nm, 488 nm, 561 nm and 635 nm. It enables the detection of up to 18 different fluorophores simultaneously which means multiple biomarkers can be detected. It has two forward scatter channels which enables cells to be

isolated based on the physical characteristics eg size and shape. The flow cytometer can isolate up to four different populations. The method of collection determines the number of populations that can be isolated (Table 4.1.1).

Method of collection	**No of populations isolated**
1.5 ml Eppendorf	1 – 4
Microscope slide	1 - 3
6 to 384 well plates	1

Table 4.1.1: Number of populations of cells isolated on the BD FACSAria™ Fusion based on the method of collection

The speed of collection is dependent upon the method of collection and the size of the nozzle on the flow cytometer. The smallest diameter circulating tumour cell identified from patient samples was 10 µm. To ensure the capture of both single circulating tumour cells and clusters, the 100 µm nozzle was selected.

A compensation matrix for the fluorophores conjugated to the antibodies was created based upon fluorescence using eComp beads ™. A gating template was created to isolate and capture the cells of interest based on their size and the presence of a nucleus. The gating template was creased based upon flow cytometry data of circulating tumour cells from patient samples, oesophageal cancer cell lines and white blood cells.

4.2: Isolation of oesophageal adenocarcinoma cells from blood

The methodology was developed with oesophageal cancer cells added to healthy donor blood. First the ability of the BD FACSAria™ Fusion sorter to enrich and purify oesophageal cancer cells from whole blood was evaluated. An additional aim of the experiment was to evaluate the methods for imaging and identifying the cells following the single cell isolation using a high resolution flow cytometer and confocal microscope.

Oesophageal adenocarcinoma cell lines which express a range of epithelial markers were selected for the experiment. OE19 cells express EpCAM and HER2, SK-GT-4 cells express EpCAM and cytokeratins and FLO1 cells express Cytokeratin only.

Cells are sorted in two stages. In the first stage, cells were isolated based upon their physical characteristics. The threshold for the minimum diameter of 10 µm for cells to be isolated was derived from data of circulating tumour cells and white blood cells from patient samples. Majority of the white blood cells are smaller than 10 µm and thus this threshold allows effective enrichment. Cells with diameter greater than 10 µm and the presence of a nucleus were selected for further analysis. This population contained both white blood cells and the oesophageal cancer cells. Figure 4.2.1 illustrates two distinct populations of cells which have been gated based upon their expression of HER2 and CD 45. Cells which express HER2 and do not express CD 45 were sorted for further analysis as illustrated in the gating template in Figure 4.2.1.

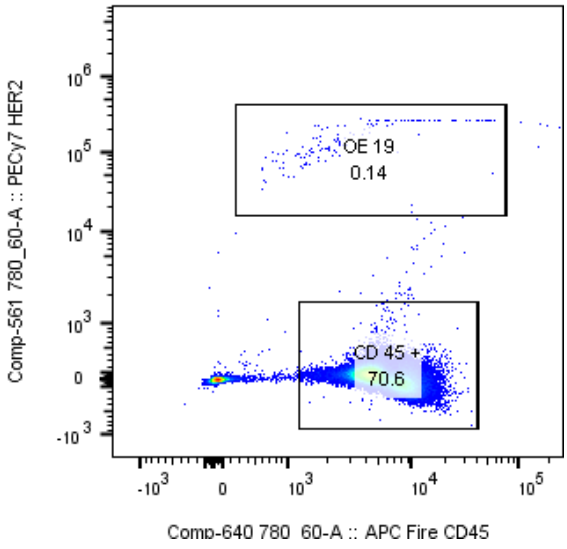


Figure 4.2.1: Isolation of individual oesophageal adenocarcinoma cells from blood based on the positive expression of epithelial human epidermal growth factor (HER2) and negative expression of CD 45.

One thousand OE19 cells were cultured in RPMI medium, trypsinised, counted and added to one ml of blood from a healthy volunteer. Red blood cells were lysed with BD Phospholyse fix buffer and permeabilised with BD phosphoflow Perm/Wash buffer for one hour at room temperature. The cell pellet was incubated with Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human (HER-2) antibody (Cat# 324414) at 1 in 100 dilution, APC-Fire™ 750 anti-human CD45 antibody (Cat # 30406) at 1 in 100 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The single cell sorting was carried out on the BD FACSAria™ Fusion flow cytometer. Cells were sorted based on the expression of the expression of PE/Cy7 conjugated anti-human CD340 (erbB2/HER-2) antibody and APC-Fire™ 750 anti-human CD45 antibody.

A total of 1500 cells were isolated based on the selection criteria. Of which, 1000 cells were collected in a 1.5 ml Eppendorf and the remaining 500 cells were placed on a microscope slide. A total of 850 cells were imaged on the high resolution imaging flow cytometer, 550 cells were positively identified as OE19 cells which express EpCAM and HER2 and absence of CD45 expression as illustrated in Figure 4.2.2. The remainder of the cells were expressed CD45 which indicates that cells isolated based on the expression of HER2 but not CD45 are contaminated with white blood cells. This demonstrated a recovery of 55% of the OE19 cells using the method for sorting and analysis. Furthermore, the flow cytometer isolated both single cells and clusters as illustrated in Figure 4.2.2

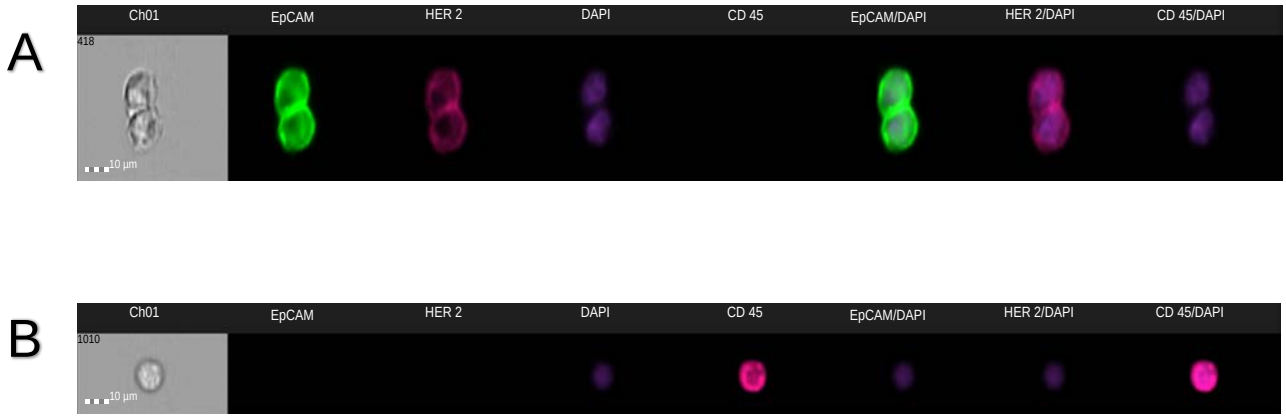


Figure 4.2.2: Characterisation by imaging flow cytometry of populations of OE19 cells isolated by FACS

A: OE19 cells B: white blood cell

The cell suspension collected in the 1.5 ml Eppendorf as described in legend 5.2.1 was visualised by imaging flow cytometry at 40x objective. The illumination intensities for the excitation lasers were set at 100, 200, 200 and 150 mW, respectively 405 nm, 488 nm, 561 nm and 658 nm wavelength. The data was analysed and images of the cells were derived using the IDEAS software

Figure 4.2.3 illustrates images of OE19 cells with a confocal microscope post single cell sorting.

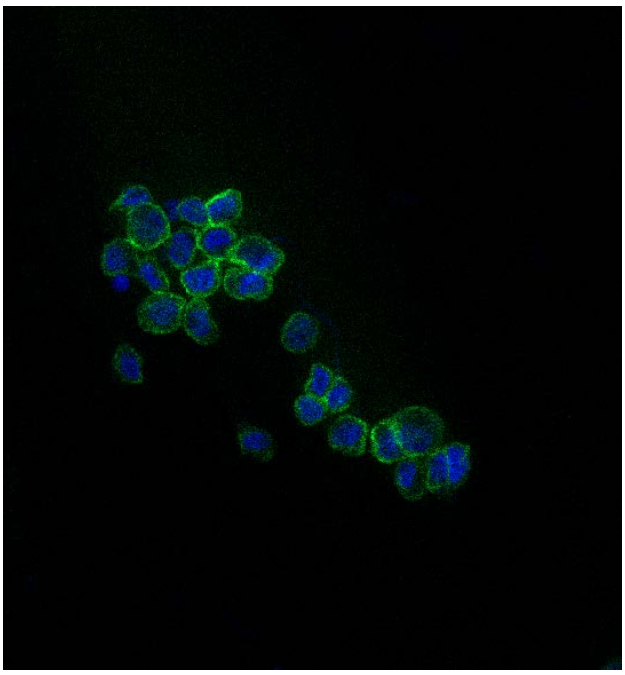


Figure 4.2.3: Visualisation of the sorted OE 19 cells on the confocal microscope

The objects isolated in Figure 4.2.1 were placed directly onto a microscope slid. Cells were visualised on Leica SP8 Confocal (Inverted) microscope. The images were taken using the 40x objective. Images were analysed on the Leica Application Suite X software

The blue colour denotes the staining of the nucleus with DAPI. The green colour denotes the immunofluorescence of the antibody conjugated to EpCAM biomarker located on the membrane of the cell.

Of the 500 objects sorted onto the microscope slide, 350 OE19 cells were identified with the confocal microscope based on the membranous expression of EpCAM and DAPI stained nuclei. Imaging of the OE 19 cells using the confocal microscope revealed a lower recovery rate at 35% when compared to 55% recovery rate with the imaging flow cytometer.

The presence of white blood cells in the sample analyzed on the high resolution flow cytometer showed that the isolated cells were contaminated with white blood cells. In addition, HER2 not detected on the OE19 cells on the confocal microscope. The cells were imaged on a Leica SP8 confocal microscope which has lasers for excitation set at 458 nm, 476 nm, 488 nm and 514 nm. The excitation peak of the PE/CY7 (erbB2/HER2) fluorophore is 565 nm and APC-Fire™ 750 fluorophore is 650 nm which were greater than the wavelength of the excitation lasers present in the confocal microscope and that means that the excitation peaks is not detectable.

4.3 Separation of oesophageal adenocarcinoma cells that express HER2 from oesophageal adenocarcinoma cells that do not

The next experiment focused on separation of two different populations of oesophageal cancer cell lines based on the differential expression of a single biomarker. SK-GT-4 cells express EpCAM and OE19 cells express both EpCAM and HER2. One hundred SK-GT-4 and One hundred OE19 cells were added to blood from a healthy human volunteer and incubated with, Alexa Fluor 488 conjugated anti-human CD326 (EpCAM), PE/Cy7 conjugated anti-human CD340 (erbB2/HER2) and APC-Fire™ 750 anti-human CD45 antibody. Figure 4.3.1 illustrates the gating template used on the FACS machine to isolate the different populations of cells based on biomarker expression.

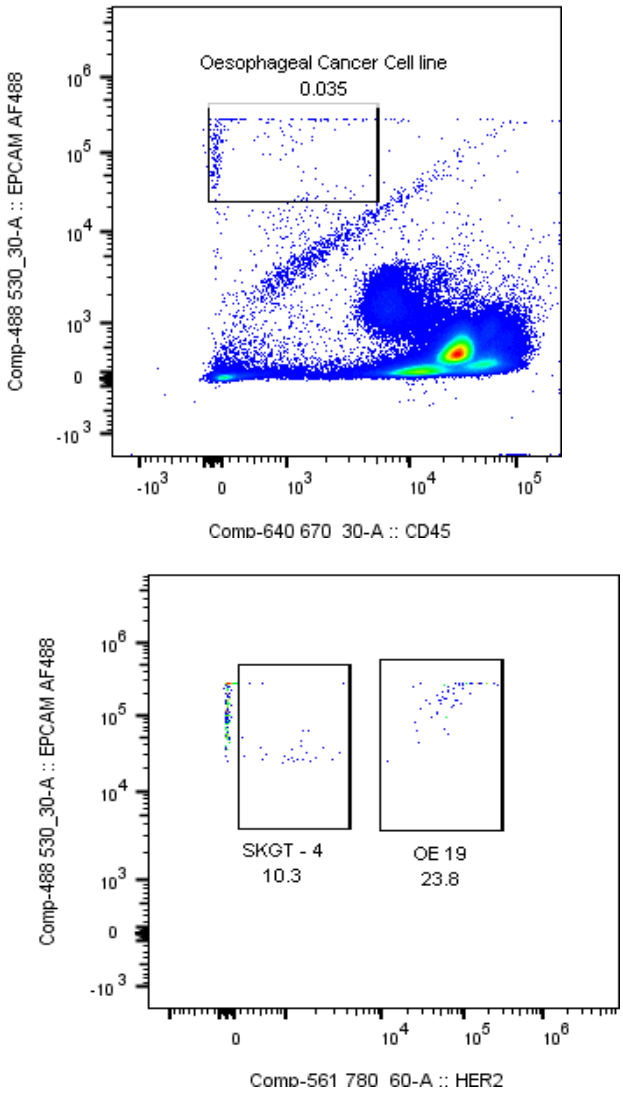


Figure 4.3.1: Isolation of individual oesophageal adenocarcinoma cells blood origination from two oesophageal cell lines from blood based on the expression of epithelial adhesion molecule (EpCAM) and human epidermal growth factor receptor 2 (HER2)

One hundred OE19 cells and one hundred SK-GT-4 cells cultured in RPMI medium were added to 1 ml of blood obtained from a healthy human volunteer Red blood cells were lysed with BD Phospholyse fix buffer and permeabilised with BD phosphoflow Perm/Wash buffer. The cells were incubated with the following anti-bodies: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, PE/Cy7 conjugated anti-human CD340 (erbB2/HER2) antibody (Cat# 324414) at 1 in 100 dilution, APC-Fire™ 750 anti-human CD45 antibody (Cat # 30406) at 1 in 100 dilution and DAPI (10 μg/ml) at 1 in 100 dilution. Cells were first isolated on the flow cytometer based on their physical characteristics. The cells were then separated based on their expression of EpCAM and CD45 (Fig 5.3.1 a). The cells with a positive expression of EpCAM and negative expression of CD 45 as illustrated by the gating template in Fig 5.3.1.a were then sorted into two populations based on their differential expression of the HER2 (Fig 5.3.1 b) and collected in two individual 1.5 ml Eppendorf tubes.

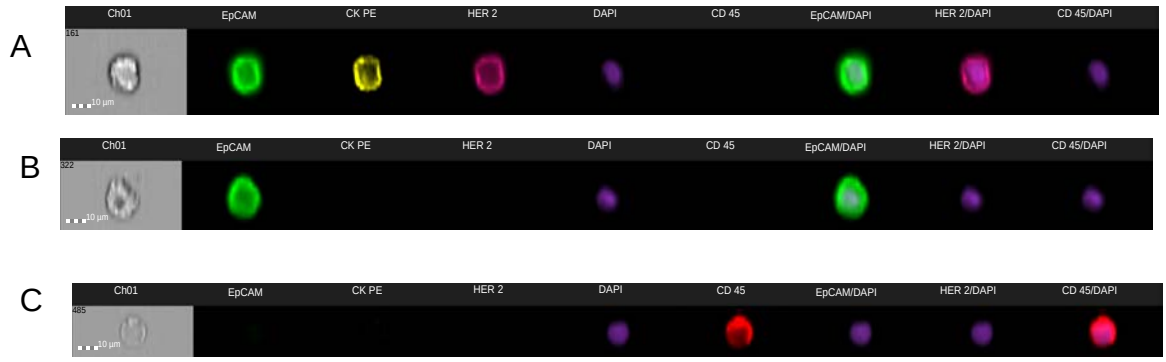


Figure 4.3.2: Characterisation by imaging flow cytometry of populations of OE19 and SK-GT-4 cells isolated by FACS

The suspension collected in the 1.5 ml Eppendorf tubes was visualised on the imaging flow cytometer at 40x objective. The wavelength of excitation lasers were 405nm, 488nm, 561nm and 658nm and the voltages set at 100, 200, 200 and 150 mW. As described in the legend to figure 5.3.1, two population of cells were separated based on the positive expression of EpCAM and negative expression of CD 45. Panel A illustrates objects isolated which expressed both EpCAM and HER2. Panel B illustrates objects isolated and collected which expressed EpCAM alone. There was evidence of contamination with white blood cells in both of the isolated cell populations as illustrated in Panel C.

As illustrated in Figure 4.3.1, it is possible with the flow cytometer to differentiate the oesophageal cancer cells and white blood cells based on the expression of EpCAM and CD45.

Further separation of the oesophageal cancer cell population is then achieved based on the differential expression of HER2 between the two cell lines. Subsequent analysis of the cell suspension on the imaging flow cytometer identified the two oesophageal cancer cell lines as illustrated in Figure 4.3.2. A total of 65 OE19 cells and 53 SK-GT-4 cells were sorted and separated in the above experiment which indicates a recovery of 64% and 53%.

4.4 Isolation and culture of oesophageal adenocarcinoma cells from whole blood

The previous experiments demonstrated that the potential of separating oesophageal cancer cells from whole blood based upon the expression of epithelial biomarkers. The next step was to develop a method that would allow the separated cells to be placed directly into culture. BD Phospholyse fix™ is the current buffer used to lyse red cells. This buffer contains formalin which fixes cells by cross linking the amino groups. This prevents cellular growth and division, thus makes it unsuitable for use when placing cells directly into culture. The aim of the experiment was to identify a red cell lysis buffer that achieves the optimum enrichment whilst preserving the viability of the oesophageal cancer cells for culture. Ammonium chloride is a buffer routinely used for red cell lysis. BD Pharm Lyse™ is chemically similar to BD Phospholyse fix™ but does not contain formalin.

Six millilitres of blood was obtained from a healthy volunteer and divided equally into six individual 14 ml Falcon tubes pre-blocked with 3% BSA. A stock solution of 1.5M ammonium chloride solution was prepared as per a standard protocol and combined with 100mM sodium bicarbonate and 1mM Na_2EDTA. The stock solution of ammonium chloride was diluted with sterile water to achieve a 1:10 ratio. Ten millilitres of the diluted ammonium chloride solution was added to two of the 14 ml Falcon tubes each containing 1 ml of blood. The solution was inverted gently ten times and then incubated in the dark at room temperature for 20 minutes. The solution was then centrifuged at 300 x g for 10 min and the supernatant was carefully discarded. The cell pellet was resuspended in 1 ml of PBS and the cells were counted using a haemocytometer. The experiment was repeated using the BD Pharm Lyse™ buffer buffer and BD Pharm Lyse™ fix buffer as per the standard manufacturer protocol. Six millilitres of blood from a healthy human volunteer was divided equally into six individual 14ml Falcon tubes which were pre-blocked for 20 minutes with 3% bovine serum albumin (BSA) at room temperature. The blood samples underwent red cell lysis as per the standard protocol for each

red cell lysis buffer. Cells from each of the individual cell suspensions were counted on a haemocytometer following the red cell lysis. The number of cells recovered from one ml of blood post red cell lysis for each individual buffer is noted in the Table 5.4.1.

Table 4.4.1: Comparing the recovery of white blood cells using different methods of red cell lysis

	No of cells post red cell lysis – A	**No of cells post red cell lysis – B**
Ammonium Chloride	7.5 x 10^5 cells	6.5 x 10^5 cells
BD Pharm Lyse™ buffer buffer	2.5 x 10^6 cells	2 x 10^6 cells
BD Pharm Lyse™ fix buffer buffer	2.25 x 10^6 cells	2.1 x 10^6 cells

The experiment indicated that the efficiency and enrichment of BD Pharm Lyse™ buffer was comparable to the BD Pharm Lyse™ fix buffer. Thus optimum enrichment could be achieved using the BD Pharm Lyse™ buffer in comparison to the Ammonium chloride solution.

The next experiment on focused on comparing the efficiency of enrichment and evaluating the viability of the tumour cells post isolation based on the expression of an epithelial biomarker. Ten thousand SK-GT-4 cells were added to 1 ml of blood. The red blood cells were lysed with the BD Pharm Lyse™ buffer as per the standard protocol under sterile conditions. The experiment was repeated with ammonium chloride to compare the enrichment and viability of oesophageal cancer cells between the two methods of red cell lysis.

The cell pellet obtained post red cell lysis was incubated with the following antibodies, Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody and Alexa Fluor 647 anti-human CD45 antibody. The oesophageal adenocarcinoma cells were isolated with a flow cytometer using the gating template derived from previous experiments as illustrated in Figure 4.4.2, cells were isolated based on their positive expression of EpCAM and negative expression of CD 45.

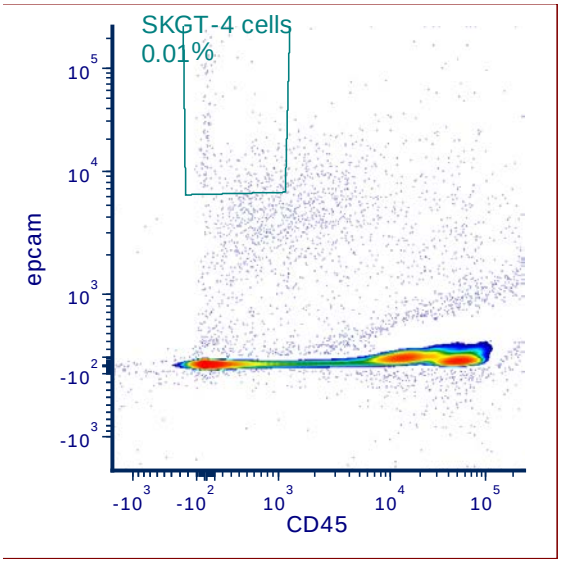
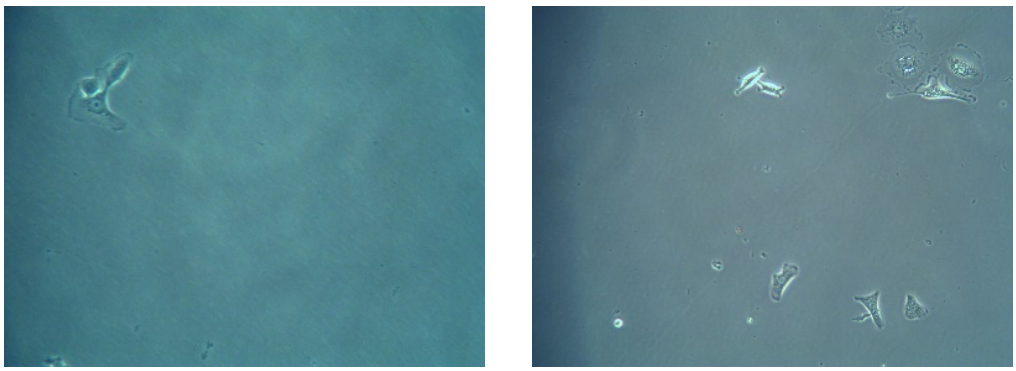


Figure 4.4.2: Isolation of oesophageal adenocarcinoma cells from blood based on the expression of epithelial adhesion molecule (EpCAM)

Ten thousand SK-GT-4 cells cultured in RPMI media were added to 1 ml of blood obtained from a healthy human volunteer. Red blood cells were lsyed with the BD Pharm Lyse™ buffer. The cell pellet post red blood cell lysis was incubated with the following antibodies: Alexa Fluor 488 conjugated anti-human CD326 (EpCAM) antibody (Cat # 324210) at 1 in 100 dilution, Alexa Fluor 647 anti-human CD45 antibody (Cat #304018) at 1 in 200 dilution and DAPI (10 µg/ml) at 1 in 100 dilution. The single cells were isolated on the BD FACSAria™ Fusion flow cytometer. Objects which expressed EPCAM and did not express CD 45 were isolated into an individual well in a six well plate containing RPMI media with 20% foetal calf serum.

As illustrated in Figure 4.4.2, two population of cells were identified. Cells which express EpCAM and do not expression CD 45 were isolated into an individual well of a six well plate containing 2 ml of RPMI media and 20% foetal calf serum.

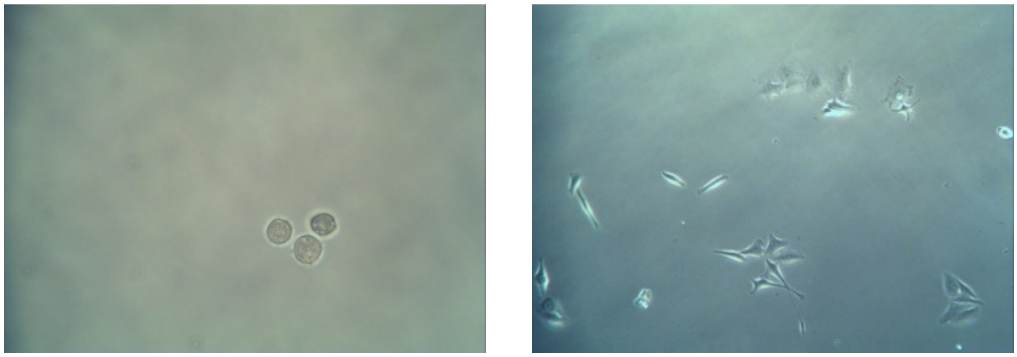
A total of 6500 objects were isolated and placed into culture from the sample in which red blood cells were lysed with the BD Pharm Lyse™ buffer. Only 2450 objects were sorted into culture from the sample in which red blood cells were lysed with the ammonium chloride buffer. Figure 4.4.3 illustrates images of SK-GT-4 cells in culture post single cell isolation following red cell lysis with ammonium chloride. Figure 4.4.4 illustrates images of SK-GT-4 cells in culture post single cell isolation following red cell lysis with BD Pharm Lyse™ buffer.



DAY 0 DAY 5

Figure 4.4.3: Images of the SK-GT-4 cells in culture post single cell isolation following red cell lysis with Ammonium Chloride

Images of the SK-GT-4 cells obtained on the light microscope on day 0 and day 5 of culture post single cell sorting



DAY 0 DAY 5

Figure 4.4.4: Images of the SK-GT-4 cells in culture post single cell isolation following red cell lysis with BD Pharm Lyse™ buffer

Images of the SK-GT-4 cells obtained on the light microscope on day 0 and day 5 of culture post single cell sorting

SK-GT-4 cells were visible and identified based on their morphology on the light microscope post single cell sorting in the six well plate as illustrated in Figure 4.4.4. In addition, the viability of the SK-GT-4 cells post single cell sorting was confirmed with the growth of the cells on Day 5 of culture. In the cohort in which red blood cells were lysed with the ammonium chloride buffer as illustrated in Figure 5.4.4, there were no visible SK-GT-4 cells present in culture on Day 0. However, there were viable SK-GT-4 cells on Day 5 of culture. The experiment highlights the feasibility of sorting viable single tumour cells based on the expression of epithelial biomarkers into culture using the BD FACSAria™ Fusion flow cytometer.

5.3 Discussion

Isolating single CTCs from blood remains a challenge due to the low concentration of CTCs being present in patients with oesophageal adenocarcinoma (128-132). In addition, the small number of CTCs isolated results in insufficient DNA and RNA being available for next generation sequencing. Optimising enrichment of CTCs from blood and reducing the contamination of white blood cells is vital. Previous studies have employed a variety of methods to isolate individual CTCs from patient's blood. Positive selection of CTCs based on the expression of epithelial markers using flow cytometry, eg CellSearch (148, 149). Dielectrophoresis has been used to isolate single CTCs in patients with breast cancer (150). Lohr et al, used a combination of immunofluorescence

and laser microdissection to isolate single CTCs in patients with metastatic prostate cancer(151). In this chapter, I have demonstrated the successful isolation of individual tumour cells from blood is possible using a combination of biomarker expression and the physical characteristics of the cell of interest eg size. The method of single cell isolation is able to achieve optimum enrichment with minimal loss with recovery rates of 53%-64%. Multiple epithelial biomarkers can be employed to isolate individual population of cells. I demonstrated the ability to isolate two populations of oesophageal adenocarcinoma cells from whole bloods based on their differential expression of HER2.

Successful culture of CTCs in the ex vivo environment unlocks a wide range of experimental possibilities. The potential to identify new biomarkers and to evaluate new therapeutic treatment regimes. CTCs in culture can be used in conjunction with animal models to better

understand the molecular mechanisms and transcription factors involved in the biology of cancer metastases. As stated before, CTCs are a rare population and methods of enrichment can damage the CTCs and reduce their viability. Previous studies have used the CTC-I chip to successfully culture CTCs in patients with metastatic breast cancer (152). This method isolates CTCs based upon their size and positive expression of EpCAM. Bobek et al, cultured CTCs from patient with oesophageal adenocarcinoma and squamous cell carcinoma (130). CTCs were isolated using a filtration based system and positively identified with immunofluorescence using epithelial biomarkers. Cells were then placed in RPMI media with 10% FBS in 5%CO_2 at 37^0C. The study reported that despite cell growth, confluence was not reached and cells became senescence after 14 days. In my study I have demonstrated successfully that oesophageal cancer cells can be isolated from blood and placed directly into culture using flow cytometry. This method achieve optimum enrichment and preserves the viability of the cancer cells as demonstrated by the presence of tumour cell growth at five days. The next stage would be to use the method to culture CTCs in patients with oesophageal adenocarcinoma.

Comparison of the molecular phenotype of the primary tumour and the isolated CTCs would aid in the understanding of origin and function of the CTCs in the development of oesophageal cancer. In addition, sequential measurement and analysis of CTCs at each stage of the treatment pathway will evaluate the response to treatment and identify prognostic factors that influence tumour progression. At present there a number of methods used to perform downstream genomic analysis of the isolated single circulating tumour cells (153). The next stage of study would be to develop a method for next generation sequencing of the isolated single circulating tumour cell in patients with oesophageal adenocarcinoma.

Chapter 5: Circulating and disseminated tumour cells in patients undergoing curative treatment for oesophageal adenocarcinoma

5.1 Introduction

Patients undergoing curative treatment for oesophagogastric cancer can be divided into three main cohorts based on the stage of disease and the treatment modality they will receive. Patients with early oesophagogastric cancer clinically staged with T2 or less and with no evidence of nodal or metastatic disease will be considered for surgery alone. Patients with clinically staged T3 or greater and/or evidence of nodal disease will be considered for peri-operative chemotherapy and surgery or surgery alone if they are unfit for multi-modality treatment. The aim of this chapter is to identify the presence the circulating and disseminated tumour cells from blood and bone marrow of patients undergoing curative treatment for oesophagogastric cancer within these three cohorts of patients. Secondary aims are to characterize the morphology of the circulating and disseminated tumour cells present and to investigate the correlations with prognostic factors derived about the resected tumours, eg presence of lymph node metastasis, tumour regression grade and presence of lymphovascular invasion. The overall objective will be to evaluate the extent and prognostic significance of circulating and disseminated tumour cells in oesophagogastric cancer. Additional objectives will be to identify prognostic biomarkers present which influence response to oncological treatment and survival in patient undergoing curative treatment for oesophagogastric cancer.

5.2 Results

5.2.1 Patient recruitment

Ethical approval for the study was obtained with the Health Research Authority NHS England (Appendix A). The inclusion criteria for the study were patients over the age of 18 undergoing curative and palliative treatment for oesophagogastric adenocarcinoma. Patients were identified at the Northern Oesophagogastric cancer MDT in the Royal Victoria infirmary, Newcastle upon Tyne. Patients eligible for the study were approached prior to their surgical treatment. The study was discussed and a patient information sheet was provided to the

patient. Patients were included into the study following completion of the consent form (Appendix B) with the principal investigator. Blood samples were obtained prior to surgery and at 48-72 hours post surgery. Bone marrow from the rib excised as a routine part of an open oesophagectomy was collected at the time of surgery. Pathological data was obtained from the resected tumour. Follow up data regarding the patients was collected from clinic visits and the Northern Oeosophagogastric unit database. Table 6.2.1.1 illustrates the demographics of the patient's recruited into the study and the clinical stage of the tumour.

Table 5.2.1.1: The demographics and the clinical stage of the tumour at presentation in patients with oesophageal cancer undergoing curative treatment

Identifier Number	Age	Sex	Tumour Site	Tumour Length	Staging - T	Staging - N	Staging - M
A1	65	F	Distal Oesophagus	3 cm	T3	N1	M0
A3	54	M	Mid-oesophagus	2 cm	T2	N0	M0
A4	59	M	Junctional	8 cm	T3	N1	M0
A5	59	M	Junctional	3 cm	T3	N1	M0
A6	69	M	Distal Oesophagus	5 cm	T3	N1	M0
A7	56	M	Distal Oesophagus	3 cm	T3	N0	M0
A8	49	M	Distal Oesophagus	2 cm	T1b	N0	M0
A9	62	M	Junctional	5 cm	T4a	N1	M0
A10	62	M	Junctional	5 cm	T4a	N1	M0
A11	68	F	Distal Oesophagus	9 cm	T3	N0	M0
A12	75	M	Distal Oesophagus	5 cm	T3	N1	M0
A13	66	F	Junctional	2 cm	T3	N2	M0
A14	66	M	Distal Oesophageal	3 cm	T3	N0	M0

A15	59	M	Junctional	2 cm	T3	N2	M0
A16	64	M	Junctional	7 cm	T4a	N1	M0
A17	60	M	Distal oesophagus	5 cm	T3	N1	M0
A18	66	M	Distal oesophagus	3 cm	T1b	Nx	Mx
A19	64	M	Distal Oesophagus	3 cm	T1b	N0	M0
A20	81	M	Distal Oesophagus	3 cm	T2	N0	M0
A21	67	M	Distal Oesophagus	8 cm	T3	N1	M0
A22	67	M	Distal Oesophagus	5 cm	T4	N0	M0
A23	73	M	Distal Oesophagus	6 cm	T4	N2	M0
A24	69	M	Junctional	7 cm	T4	N1	M0
A25	73	M	Distal Oesophagus	3 cm	T1b	N0	M0
A26	65	M	Distal Oesophagus	6 cm	T3	N1	M0
A27	62	M	Distal Oesophagus	3 cm	T3	N0	M0
A28	49	M	Distal Oesophagus	5 cm	T3	N2	M0
A29	56	M	Distal Oesophagus	2 cm	T1a	N0	M0
A30	80	M	Junctional	6 cm	T3	N0	M0
A31	66	M	Junctional	5 cm	T3	N0	M0
A32	66	M	Distal Oesophageal	7 cm	T3	N1	M0
A33	67	M	Junctional	5 cm	T3	N2	M0
A34	70	M	Distal Oesophageal	6 cm	T1a	N0	M0
A35	67	M	Junctional	6 cm	T4	N2	M0
A36	75	M	Distal Oesophagus	3 cm	T3	N3	M0

A37	57	M	Distal Oesophagus	6 cm	T3	N2	M0
A38	59	M	Distal Oesophagus	4 cm	Tx	Nx	M0
A39	65	M	Mid Oesophagus	4 cm	T3	N3	M0
A40	70	M	Distal Oesophagus	5 cm	T3	N1	M0

* The clinical and pathological stage are based on the 8th edition AJCC guidelines.

A total of 39 patients undergoing planned curative resection for oesophageal adencocarcinoma were recruited into the study between 1st November 2016 to the 23rd August 2018. Patients underwent a standardized staging protocol and findings of which were discussed in a multi-disciplinary team. Patient factors (eg medical co-morbidities) combined with the clinical stage of the tumour determined the subsequent treatment modality. One patient, A2 was excluded from the study after histology confirmed the diagnosis of squamous cell cancer made with biopsy specimen post recruitment. There are three cohort of patients as stated before. Tables 5.2.1.2, 5.2.1.3 and 5.2.1.4 list the clinical and pathological stages of the tumours, operative approach and the oncological outcomes post resection for patient in each of the three cohorts.

Table 5.2.1.2: Surgical and oncological outcomes for patients with clinical stage I and II cancer who underwent curative surgery alone

Identifier Number	Clinical Stage T*	Clinical Stage N*	Surgical Approach	Path Stage T*	Path Stage N*	No of positive lymph nodes	Total no of lymph nodes	Lymphovascular invasion
A3	T2	N0	Open	T1a	N0	0	41	No
A8	T1b	N0	Open	T1a	N0	0	39	No
A18	T1b	N0	Thoracoscopic	T1a	N0	0	34	No
A19	T1b	N0	Thoracoscopic	T1a	N0	0	36	No
A20	T2	N0	Open	T2	N1	2	54	Yes

A25	T1b	N0	Thoracoscopic	T1a	N0	0	36	No
A29	T1a	N0	Open	T1a	N0	0	20	No
A34	T1a	N0	Open	T1b	N0	0	25	No
A38	T1a	N0	Open	T1a	N0	0	48	No

* The clinical and pathological stage are based on the 8th edition AJCC guidelines.

Patients with clinical stage I and II cancer based on clinical staging underwent curative surgery alone. Three of the nine patients underwent a minimally invasive approach for the thoracic stage of the oesophagectomy. Three of the nine patients who had single modality based upon the clinical stage underwent a thoracoscopic oesophagectomy and as a consequence a rib sample was not obtained for these patients. Patient A20 was initially staged with T2 disease without nodal mestatases. However at the time of surgery there was evidence of nodal metastases (Table 5.2.1.2). This case illustrates the challenge in accurately staging nodal disease with the current modalities.

Table 5.2.1.3: Surgical and oncological outcomes for patients with clinical stage III and III cancer who underwent curative surgery alone

Identifier Number	**Clinical Stage T***	**Clinical Stage N***	**Surgical Approach**	**Path Stage T***	**Path Stage N***	**No of positive lymph nodes**	**Total no of lymph nodes**	**Lymphovascular invasion**
A7	T3	N0	Open	T3	N1	1	42	No
A21	T3	N1	Open	T3	N3	16	50	Yes
A30	T3	N0	Open	T3	N1	2	25	Yes
A36	T3	N3	Open	T3	N3	22	48	Yes

* The clinical and pathological stage are based on the 8th edition AJCC guidelines.

At initial clinical staging only two of the four patients who had single modality treatment for locally advanced cancer because they were unfit for peri-operative chemotherapy had evidence of nodal disease. However, all the patients in this cohort had evidence of nodal disease in the resected specimen. In addition, two patients had evidence of nodal metastasis in over 30% of the nodes resected.

Table 5.2.1.4: Surgical and oncological outcomes for patients with clinical stage III and IV cancer who underwent peri-operative chemotherapy and surgery

Identifier Number	Clinical Stage T*	Clinical Stage N*	Peri-operative chemotherapy regime**	Cycle of chemotherapy completed	Surgical Approach	Path Stage T*	Path Stage N*	No of positive lymph nodes	Total no of lymph nodes	Lymphovascular invasion
A1	T3	N1	ECX	2	Open	T3	N2	3	36	Yes
A4	T3	N1	ECX	3	Open	T3	N2	4	50	yes
A5	T3	N1	ECX	1	Open	T3	N2	4	34	Yes
A6	T3	N1	ECX	1	Open	T3	N0	0	25	No
A9	T4a	N1	ECX	3	Open	T3	N1	1	50	No
A10	T4a	N1	ECX	3	Open	T3	N0	0	22	No
A11	T3	N0	ECX	3	Open	T2	N0	0	24	No
A12	T3	N1	ECX	3	Open	T2	N1	1	33	No
A13	T3	N2	ECX	2	Open	T4a	N1	1	49	Yes
A14	T3	N0	ECX	3	Open	T3	N1	1	27	Yes
A15	T3	N2	ECX	3	Thoracoscopic to open	T4a	N0	0	53	Yes
A16	T4a	N1	ECX	3	Open	T1b	N0	0	41	No
A17	T3	N1	ECX	3	Open	T4a	N3	10	43	Yes

A22	T4	N0	ECX	3	Open	T3	N2	5	32	Yes
A23	T4	N2	ECX	3	Open	T0	N0	0	98	No
A24	T4	N1	ECX	3	Open	T3	N1	1	36	No
A26	T3	N1	Neo-AGIS	3	Open	T3	N3	10	30	Yes
A27	T3	N0	ECX	3	Open	T3	N2	3	29	Yes
A28	T3	N2	ECX	3	Open	T3	N3	9	33	Yes
A31	T3	N0	ECX	3	Open	T1b	N0	0	24	No
A32	T3	N1	OEO2	2	Open	T3	N0	0	34	Yes
A33	T3	N2	ECX	3	open	T1b	N2	4	20	No
A35	T4	N2	ECX	3	Open and close					
A37	T3	N2	ECX	3	Open	T3	N1	1	111	No
A39	T3	N3	ECX	3	Open	T3	N3	7	78	Yes
A40	T3	N1	ECX	3	Open	T3	N2	5	24	No

* The clinical and pathological stage are based on the 8th edition AJCC guidelines

** ECX chemotherapy regime (epirubicin, cisplatin, capecitabine). One patient was enrolled into the NEO-AGIS trail and was randomized to the peri-operative chemotherapy arm. One patient had cardiac co-morbidities and was unsuitable for the ECX (epirubicin, cisplatin, capecitabine) regime and hence received the OEO2 (cisplatin, fluorouracil) regime.

A total of 25 patients underwent perioperative chemotherapy followed by surgery. Over 80% of the patients completed the peri-operative chemotherapy regime. Two patients received only one cycle of chemotherapy and three received two cycles of chemotherapy due to intolerance to the regime. Patients underwent a CT scan post peri-operative chemotherapy to assess the response of the primary tumour and lymph nodes to the treatment. Response evaluation criteria in solid tumours (RESCIST) was used to grade the response of the tumour to the chemotherapy regime. In three patients there was evidence of tumour progression following peri-operative chemotherapy. There was evidence of tumour regression in 32% (8) of patients

who received peri-operative chemotherapy. Patient 35 had evidence of liver metastases at the time of surgery and thus did not undergo resection. Patient A23 had completed pathological response with no tumour being visible in the specimen. Only 28% (7) of patients had pathological evidence of tumour regression, TRG 3 or less at the time of resection. The majority, 72% (18) patients had tumours which had either a poor or no response to the peri-operative chemotherapy regime.

5.2.2 Circulating tumour cells in the pre and postoperative blood samples in patients undergoing curative resection for oesophagogastric cancer

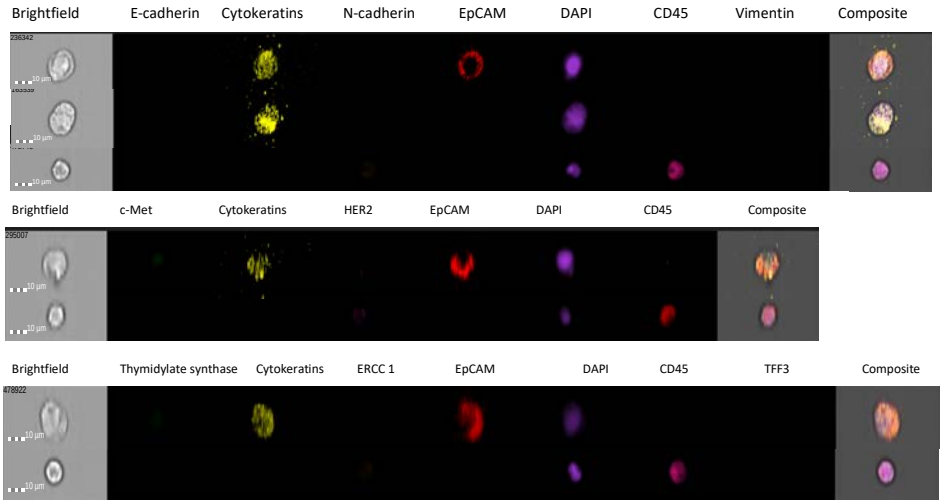
Circulating tumour cells in patients with oesophageal adenocarcinoma with the CellSearch^{R} test. Konzalla et al evaluated CTCs in patients with non metastatic oesophageal adenocarcinoma (128). They identified CTCs in only 15 of 76 patients and reported that the presence of five or more CTCs was associated with poor survival. The CellSearch^{R} test identifies a cell as a CTC if it meets the following criteria: positive expression of EpCAM, cytokeratins 8, 18 and/or 19 and negative expression of CD 45. This method does not take into account the morphological characteristics of CTCs, eg size, hyerploidy and the expression of other biomarkers, eg HER2, c Met. In addition, a cell is only classified as a CTC if it expressed both epithelial biomarkers, EpCAM and cytokeratins 8, 18+, and/or 19. CTCs which express which express only one of the epithelial biomarkers are excluded. Previous studies have demonstrated the presence of heterogeneity within the CTC population in patients with other solid organ tumours (154). In addition, there is evidence of heterogeneity within the cells in the primary tumour and lymph nodes of patients with oesophageal cancer (146). The CellSearch^{R} test potentially underestimates the number of CTCs present and is unable to assess for heterogeneity of CTCs present within the blood.

As described before, blood samples were obtained from patients 4-6 hours prior to surgery and between 48-72 hours postoperatively. Blood samples were processed in accordance with the CTC enumeration protocol and labelled with the antibodies as described in the material and methods chapter. The samples were analysed on the Imagestream^{R} high resolution imaging flow cytometer. There were three populations of cells were identified and classified as CTCs. CTCs that expressed biomarkers were divided into two populations. The first population of CTCs which expressed EpCAM and cytokeratins and the second population of CTCs which

expressed either one or more biomarkers present in the panel. CTCs which were biomarker positive consisted of 69% of the cells present. The third population of cells classified as CTCs did not express any of the biomarkers present in the panels but were morphologically different from white blood cells. This population of CTCs were larger in size and had increased nuclear content in comparison to the white blood cells present in the samples. CTCs were detected in all patients underwent curative treatment for oesophageal cancer and the following Figures 5.2.2.1, 5.2.2.2 and 5.2.2.3 illustrate the images of the CTCs detected.

A39 – T3N3 – Peri-operative chemotherapy and surgery

CTCs in pre-operative sample



CTCs in postoperative sample

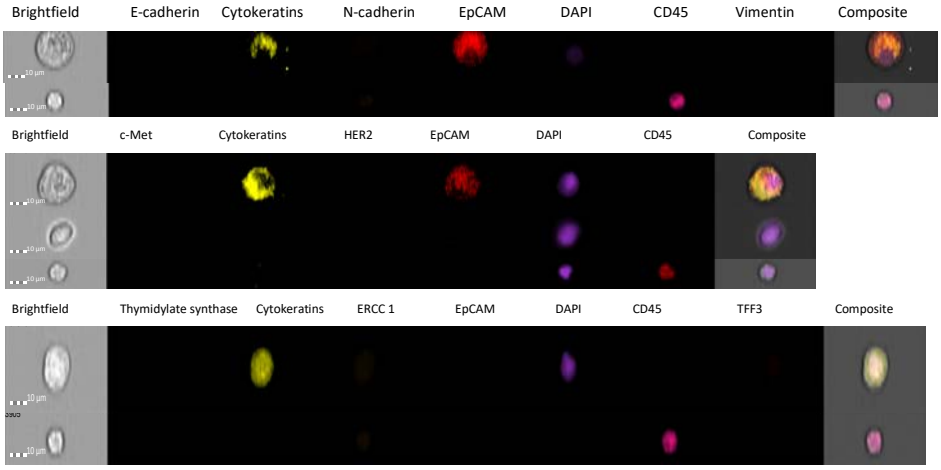


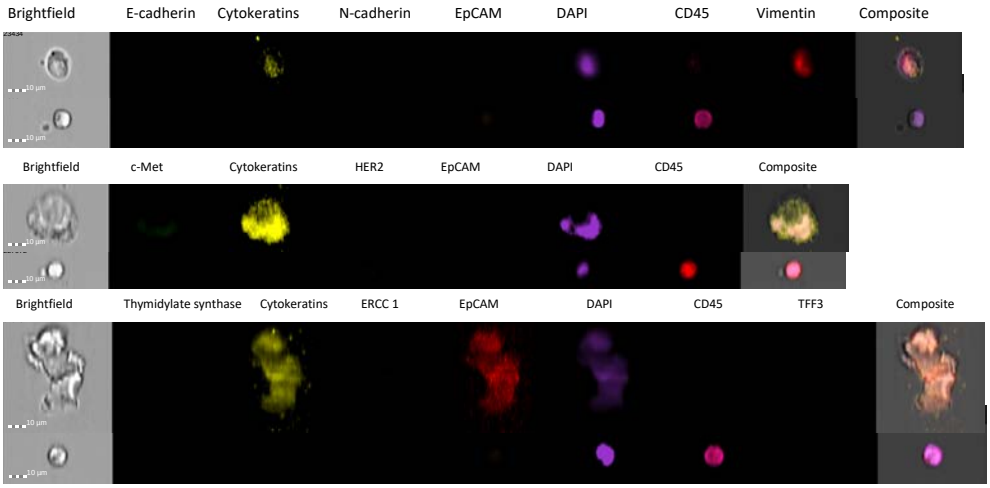
Figure 5.2.2.1: Images of CTCs detected on the high resolution imaging flow cytometer in Patient A39 who received three cycles of ECX and underwent an open oesophagectomy for clinically staged T3N3 oesophageal adenocarcinoma.

A: CTCs detected in the pre-operative sample B: CTCs detected in the postoperative sample.

In patient A39, 159 CTCs were detected in the pre-operative sample. There were two populations of CTCs present. One population of CTCs which expressed EpCAM and cytokeratins and accounted for 125 CTCs per 7.5 ml of blood. The second population expressed cytokeratins alone and accounted for 34 CTCs per 7.5 ml of blood. In the postoperative sample, the number of CTCs were higher than those present in the pre-operative sample 200 CTCs per 7.5 ml of blood versus 159 CTCs per 7.5 ml of blood. Three population of CTCs were present. Majority of the CTCs (158) expressed EpCAM and cytokeratins. The second population of CTCs expressed cytokeratins and accounted for 13 CTCs per 7.5 ml of blood. The third population of CTCs did not express any of the biomarkers in the panels but were morphologically similar to CTCs and accounted for 24 CTCs per 7.5 ml of blood.

A36 – T3N3 – Surgery Alone

CTCs in pre-operative sample



CTCs in postoperative sample

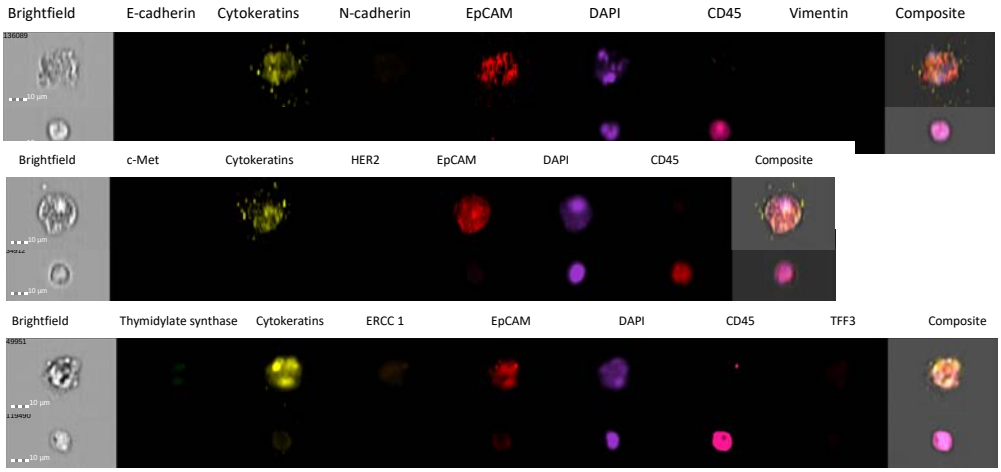


Figure 5.2.2.2: Images of CTCs detected on the high resolution imaging flow cytometer in Patient A36 who underwent an open oesophagectomy for clinically staged T3N3 oesophageal adenocarcinoma.

A: CTCs detected in the pre-operative sample B: CTCs detected in the postoperative sample.

In patient A 36, 33 CTCs per 7.5 ml of blood detected in the pre-operative sample. Nine CTCs per 7.5 ml of blood expressed Vimentin and cytokeratins and the remainder expressed EpCAM and cytokeratins. In the postoperative sample, all 52 CTCs per 7.5 ml of blood detected expressed EpCAM and cytokeratins.

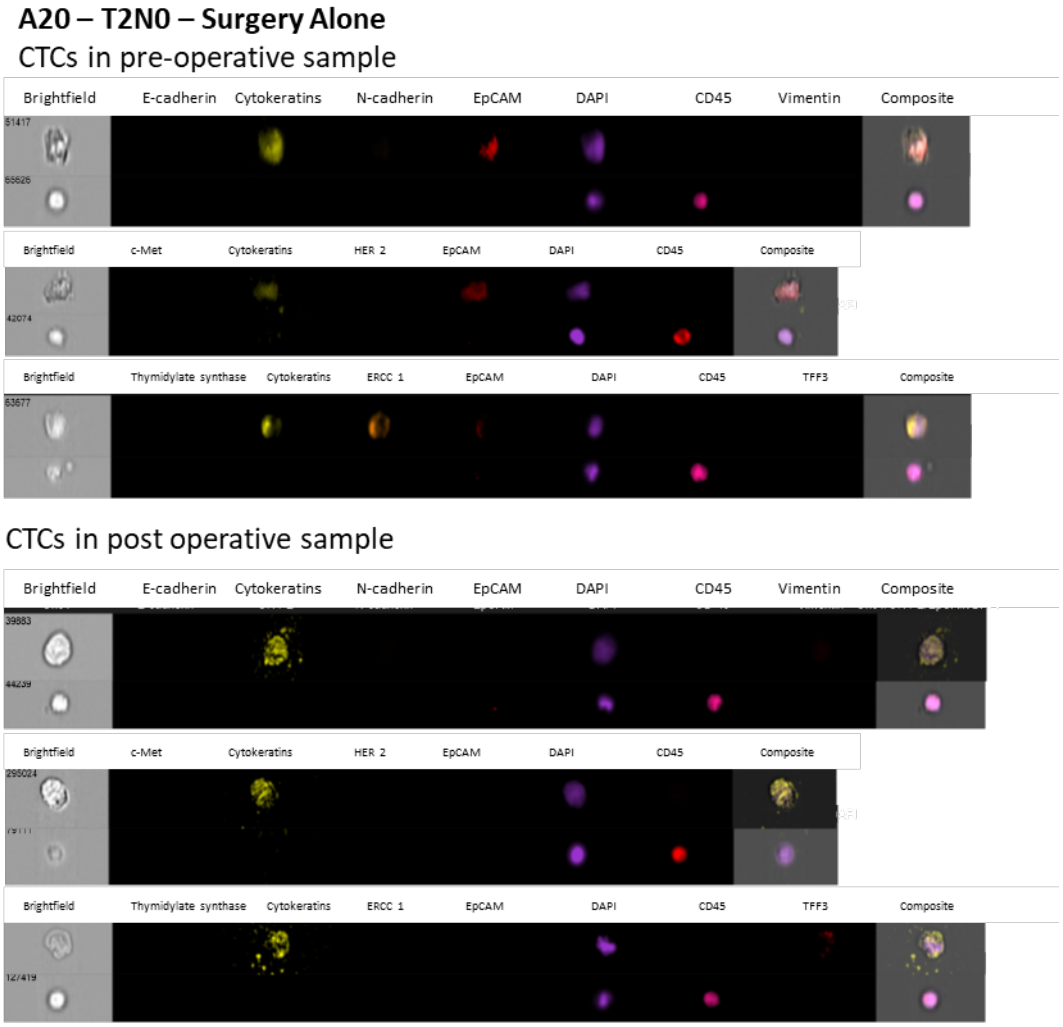


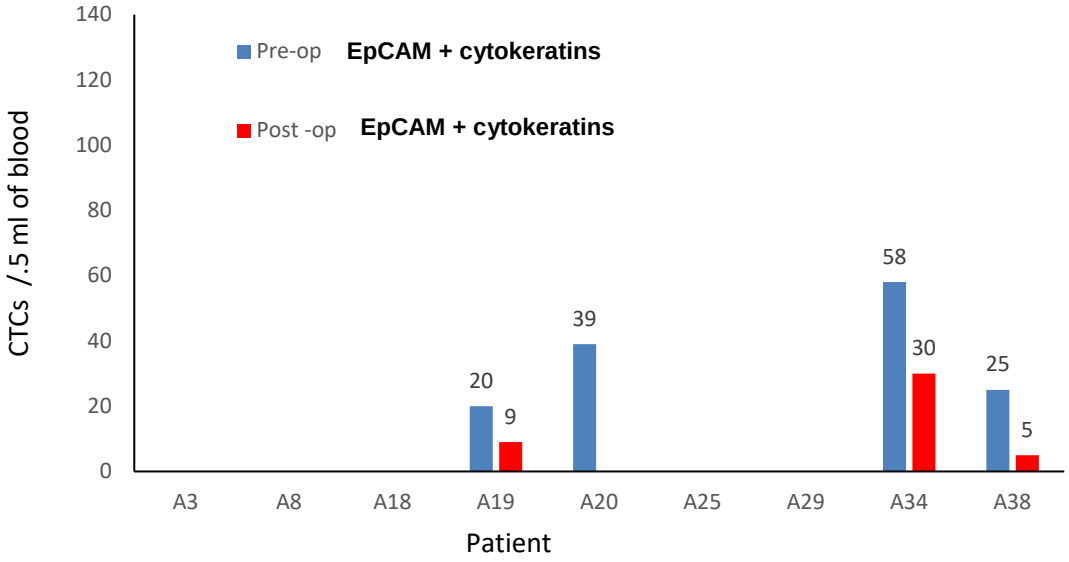
Figure 5.2.2.3: Images of CTCs detected on the high resolution imaging flow cytometer in Patient A20 who underwent an open oesophagectomy for clinically staged T2N0 oesophageal adenocarcinoma.

A: CTCs detected in the pre-operative sample B: CTCs detected in the postoperative sample.

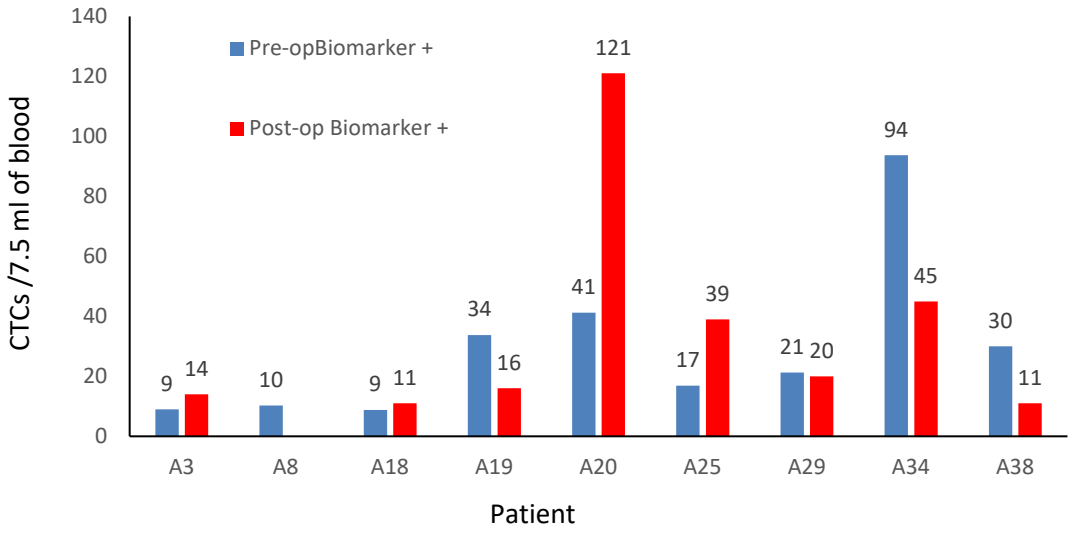
In patient A20, 41 CTCs per 7.5 ml of blood were detected in the pre-operative sample of which 39 expressed EpCAM and cytokeratins and the remainder expressed cytokeratins and ERCC 1. In the postoperative sample, 121 CTCs per 7.5 ml of blood were present and all expressed cytokeratins only.

The following figures illustrate the number of CTCs present in the pre and postoperative sample detected based on the clinical stage of the disease at the time of curative treatment and the biomarker expression of CTCs present. Figure 6.2.2.4 illustrates the number of CTCs detected in patients with early oesophageal cancer undergoing curative resection based on the presence of biomarker expression.

A


Pre-op EpCAM + cytokeratins
Post -op EpCAM + cytokeratins
CTCs /.5 ml of blood
140
120
100
80
60
40
20
0
20
9
39
58
30
25
5
A3
A8
A18
A19
A20
A25
A29
A34
A38
Patient

B


Pre-opBiomarker +
Post-op Biomarker +
CTCs /7.5 ml of blood
140
120
100
80
60
40
20
0
9
14
10
9
11
34
16
41
121
17
39
21
20
94
45
30
11
A3
A8
A18
A19
A20
A25
A29
A34
A38
Patient

C

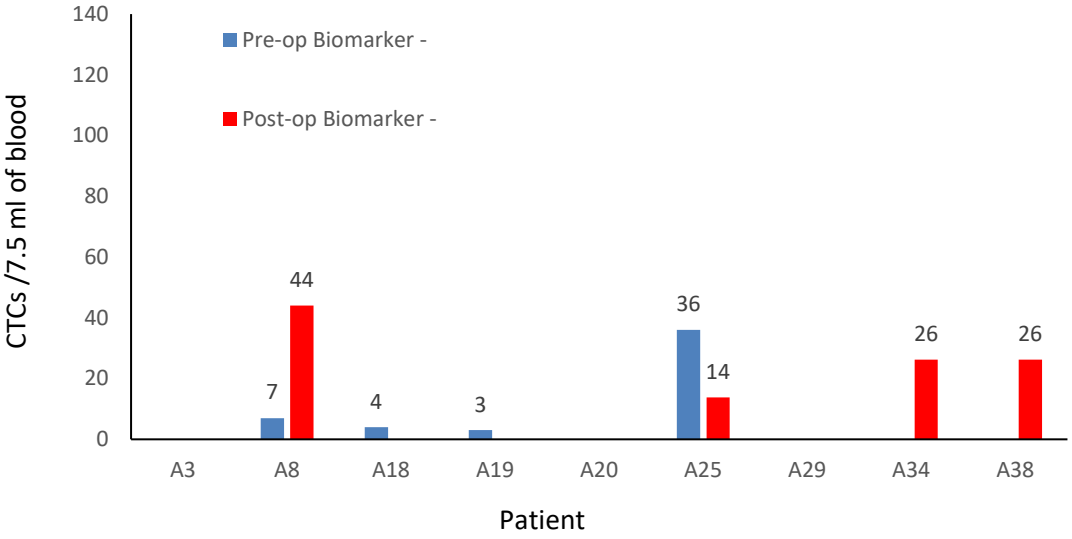
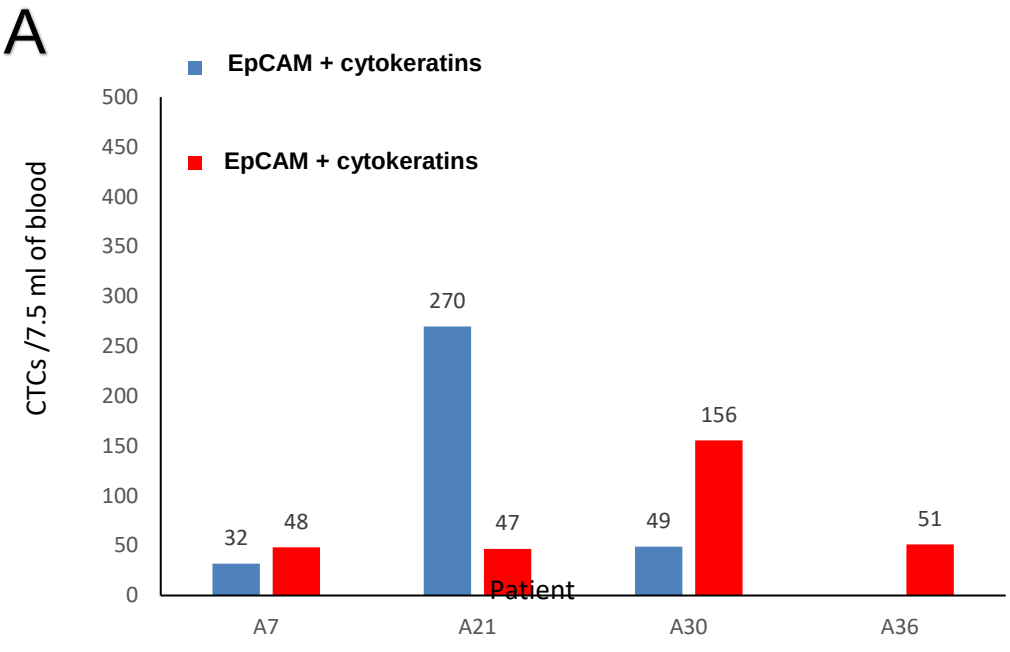

Pre-op Biomarker -
Post-op Biomarker -
CTCs /7.5 ml of blood
140
120
100
80
60
40
20
0
7
44
4
3
36
14
26
26
A3
A8
A18
A19
A20
A25
A29
A34
A38
Patient

Figure 5.2.2.4 – The number of circulating tumour cells (CTC) in the pre-operative and postoperative blood sample in patients with Stage I and II cancer in 7.5 ml of blood based on biomarker expression.

A: CTCs expressing EpCAM and CK biomarkers as per the CellSearch^{R} selection criteria B: CTCs expressing EpCAM, CK, HER2, cMET, E-cadherin, N-cadherin, Thymidylate synthase, Vimentin C: Cells which are morphologically similar to CTCS but negative for epithelial and mesenchymal markers in the antibody panel and negative for the CD 45 biomarker.

All the patients had CTCs which were biomarker positive detected in either the pre and/or postoperative sample. There were six patients with early oesophageal adenocarcinoma who had cells which were biomarker negative. Of note, CTCs which expressed both EpCAM and cytokeratins were detected in only 4 out of 9 patients. Positive identification of CTCs based on the expression of both EpCAM and cytokeratins would have underestimated the number of CTCs present. With patient A19, approximately half the CTCs expressed both EpCAM and cytokeratins. In patient A20 the rise in the number of postoperative CTCs would not have been detected if CTCs were selected based on the expression of both EpCAM and cytokeratins. Approximately 67% of cells identified were biomarker negative and met the morphological criteria of CTCs in this group of patients.

Figure 5.2.2.5 illustrates the number of CTCs detected in patients with locally advanced oesophageal cancer who underwent surgery alone based on biomarker expression.



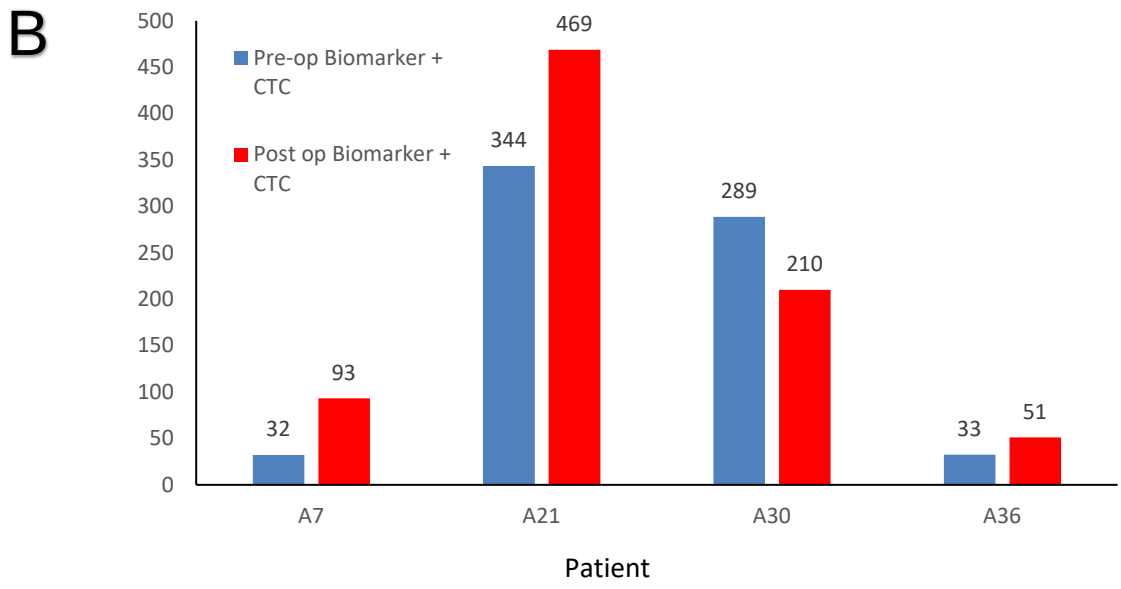


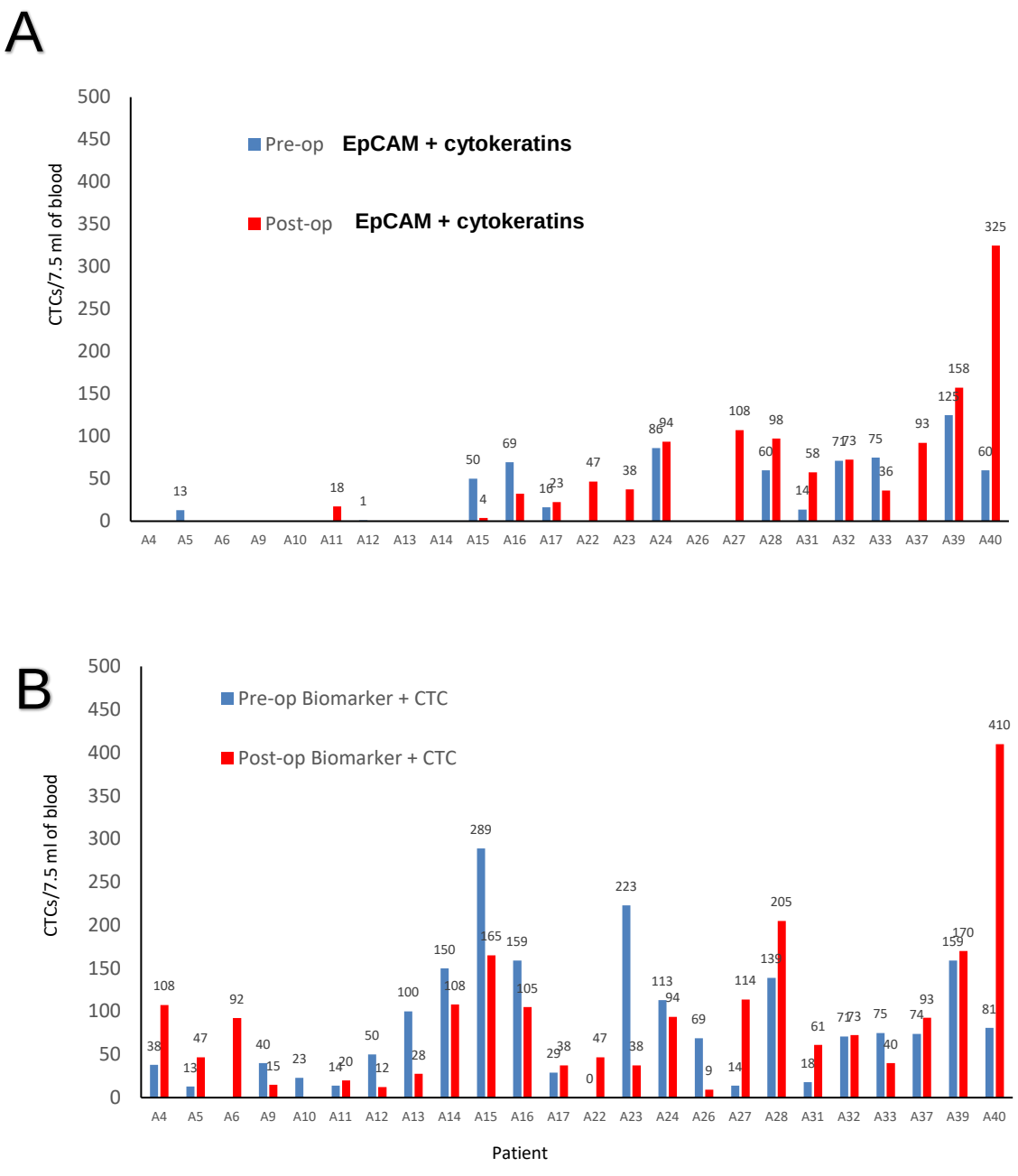
Figure 5.2.2.5 – The number of circulating tumour cells (CTC) in the pre-operative and postoperative blood sample in patients with Stage III and IV cancer who underwent surgery alone in 7.5 ml of blood.

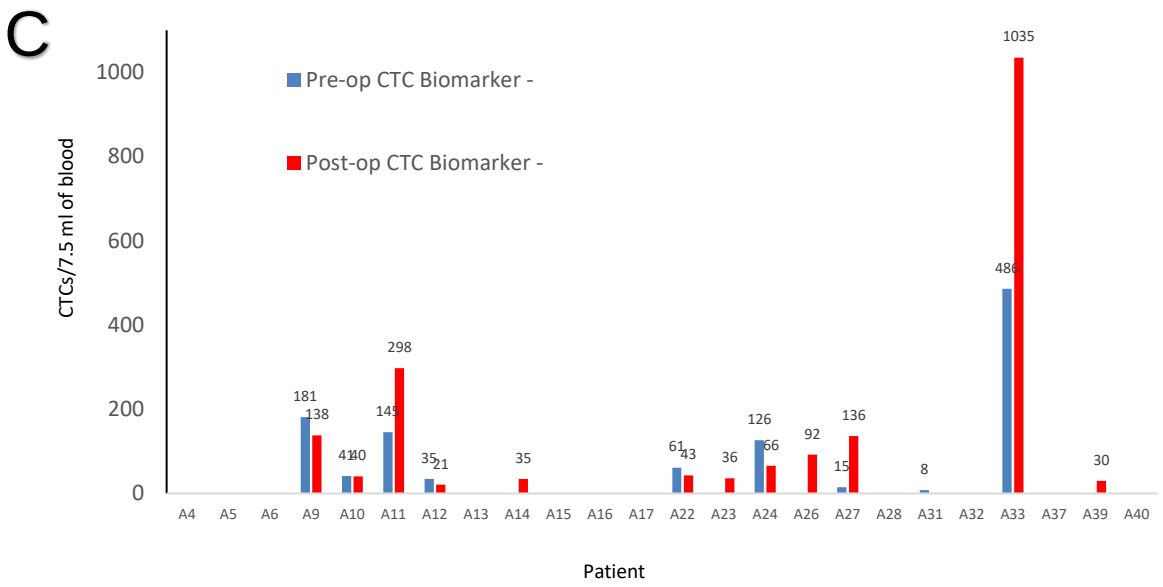
A: CTCs expressing EpCAM and CK biomarkers as per the CellSearch^{R} selection criteria B: CTCs expressing EpCAM, CK, HER2, cMET, E-cadherin, N-cadherin, Thymidylate synthase, Vimentin

In patient A21, the total number of CTCs detected was over 300 per 7.5 ml of blood in the pre and postoperative sample. Positive identification of CTCs based on the expression of both EpCAM and cytokeratins would have underestimated the number of CTCs present in all 4 patients. In patient A30, the total number of CTCs detected was lower in the postoperative sample based on all biomarker expression. In this patient, the number of CTCs which met the expressed both EpCAM and cytokeratins increased in the postoperative sample. Furthermore, in three out of four patients the number of CTCs detected which expressed both EpCAM and cytokeratins increased in the postoperative sample. This difference in biomarker expression in CTCs between the pre and postoperative samples detected supports the theory of

heterogeneity within the tumour cell population and raises the possibility of distant metastatic sites as a source of the CTCs. None of the patient had biomarker negative cells in this cohort.

Figure 5.2.2.6 illustrates the number of CTCs detected in patients with locally advanced oesophageal cancer who received peri-operative chemotherapy and surgery based on biomarker expression. A pre-operative blood sample was not obtained for patient A6 due to medical reasons. Patient A 35 did not undergo a curative resection due to the presence of metastatic disease at the time of surgery and no postoperative sample was obtained.





*Patient A35 excluded from final analysis

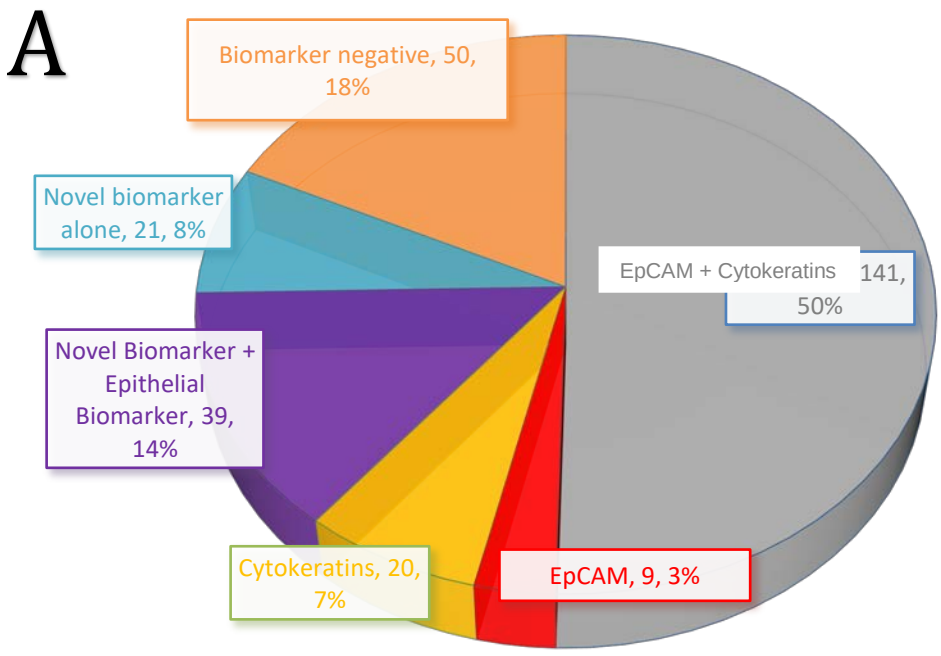
Figure 5.2.2.6 – The number of circulating tumour cells (CTC) in the pre-operative and postoperative blood sample in patients with Stage III and IV cancer who underwent peri-operative chemotherapy and surgery in 7.5 ml of blood.

A: CTCs expressing EpCAM and CK biomarkers as per the CellSearch^{R} selection criteria B: CTCs expressing EpCAM, CK, HER2, cMET, E-cadherin, N-cadherin, Thymidylate synthase, Vimentin C: Cells which are morphologically similar to CTCS but negative for epithelial and mesenchymal markers in the antibody panel and negative for the CD 45 biomarker.

There is a wide variation in the number of CTCs based on all biomarker expression detected in the pre-operative blood sample. Of note, the number of CTCs detected in some patients is comparable to the cohort of patients with early cancer. This could be attributed to the impact of peri-operative chemotherapy which downstages the cancer. CTCs which expressed both EpCAM and cytokeratins would only have been detected in 58% (14/24) patients in both the pre and postoperative samples. Of interest, this cohort of patients had the highest number of CTCs which were biomarker negative. In patients A11 and A33, over 200 of these cells per 7.5 ml of blood were detected in in the postoperative sample.

5.2.3 Characterization of circulating tumour cells detected in patients undergoing curative resection for oesophagogastric cancer

Characterizing the heterogenity within the tumour cells is of importance in understanding the molecular mechanisms of tumour evolution and to identify potential targets for therapeutic intervention. Previous studies have relied upon the CellSearch^{R} criteria which positively identifies a CTC based on the expression of both EpCAM and cytokeratins. This potentially results in only a proportion of CTCs present to be identified. In the study, eleven different biomarkers were used including EpCAM and cytokeratins to characterize the CTCs. A CTC was identified as biomarker positive if it expressed either one or more of the biomarkers present in the panel. CTCs were grouped into five individual populations based on biomarker expression. Figure 5.2.3.1 illustrates the heterogeneity within the CTCs detected in patients with Stage I and II oesophageal cancer who underwent surgery alone.



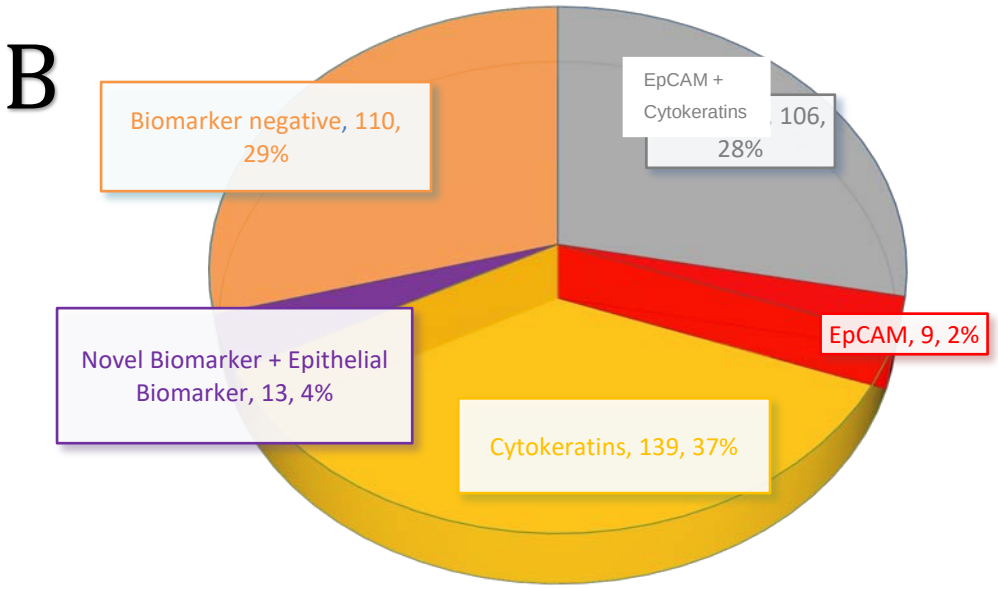


Figure 5.2.3.1 – Heterogeneity of biomarker expression in CTCs detected in patients undergoing surgery alone for Stage I and II cancer.

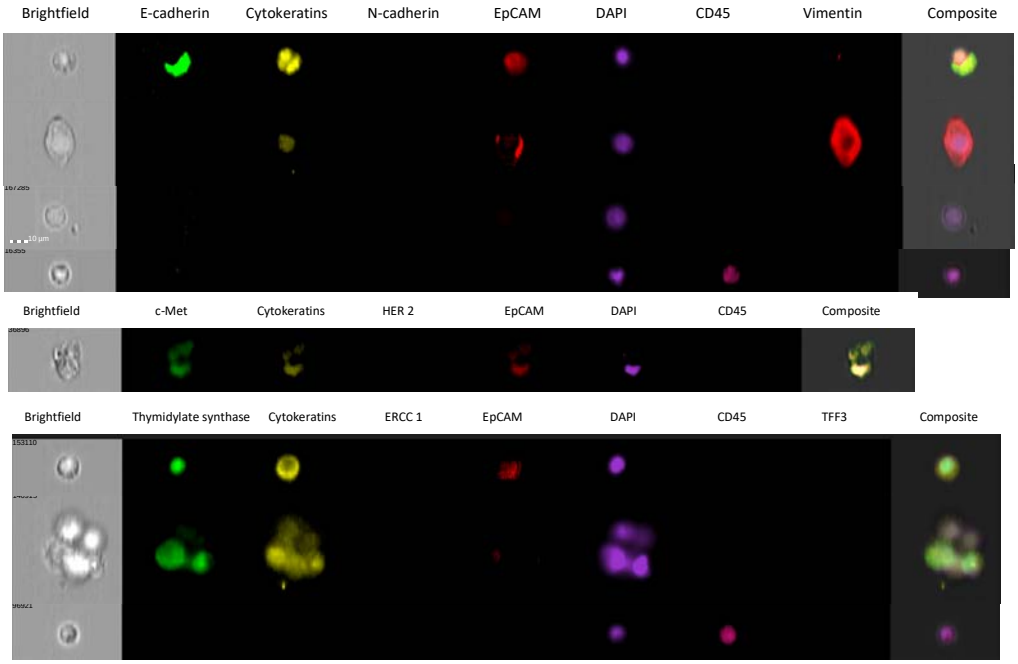
Biomarker expression in differentiated into five groups: EpCAM and Cytokeratin expression as per CellSearch^{R} selection criteria, EpCAM alone, cytokeratin alone, Novel biomarkers +/- EpCAM +/- cytokeratins– HER2, c-Met, Vimentin, ERCC1, Thymidylate synthase, E-cadherin, N-cadherin, TFF3, Novel biomarkers - HER2, c-Met, Vimentin, ERCC1, Thymidylate synthase, E-cadherin, N-cadherin, TFF3 alone and CTCs which did not express any biomarker. A – CTCs detected in the pre-operative blood sample. B- CTCs detected in the postoperative sample at 48-72 hours.

In this cohort of patients there was variation in the expression of biomarkers between the pre and postoperative blood sample. In the pre-operative blood samples, 50% of the CTCs expressed both EpCAM and cytokeratins, but this was the case in only 28% in the postoperative sample. There was a rise in the number of CTCs expressing cytokeratins. Less than 10% of CTCs expressed cytokeratins in the pre-operative sample. However, over 1/3 of the CTCs detected in the postoperative blood sample expressed cytokeratins. There was a rise in the number of biomarker negative cells between the pre and postoperative sample, 18% to 28% respectively.

Patient A19 underwent a thoracoscopic oesophagectomy for T1b oesophageal adenocarcinoma. Figure 5.2.3.2 illustrates CTCs detected in patient A19.

A19 – T1bN0 – Surgery alone – Thoracoscopic oesophagectomy

CTCs in pre-operative sample



CTCs in postoperative sample

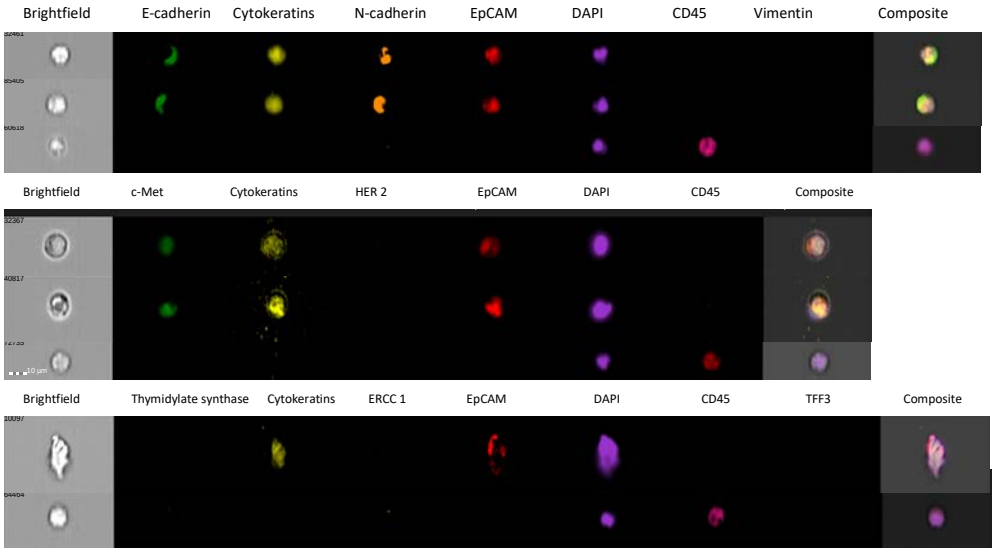


Figure 5.2.3.2 – Images of CTCs detect in Patient A19 who underwent a thoracoscopic oesophagectomy for Stage I oesophageal cancer.

A – Images of CTCs detected in the pre-operative blood sample. B – Images of CTCs detected in the postoperative blood sample at 48 hours

Patient A19 had two of the sample population of CTCs which expressed novel biomarkers in both the pre and postoperative sample. CTCs which expressed c-Met and N-cadherin were present in both the pre and postoperative sample. However, CTCs which expressed

thymidylate synthase was only present in the pre-operative sample. Of interest, the CTCs which expressed thymidylate synthase were present in clusters unlike the other CTCs present. There were biomarker negative CTCs present in the pre-operative sample but none in the postoperative sample. CTCs which expressed E-cadherin were present only in the postoperative sample. The presence of N-cadherin and E-cadherin expression in the CTCs in the postoperative sample indicate their origin from a distant site of possible metastases.

Table 5.2.3.3 illustrates the heterogeneity of the novel biomarkers present in the CTCs within this cohort in the pre and postoperative sample.

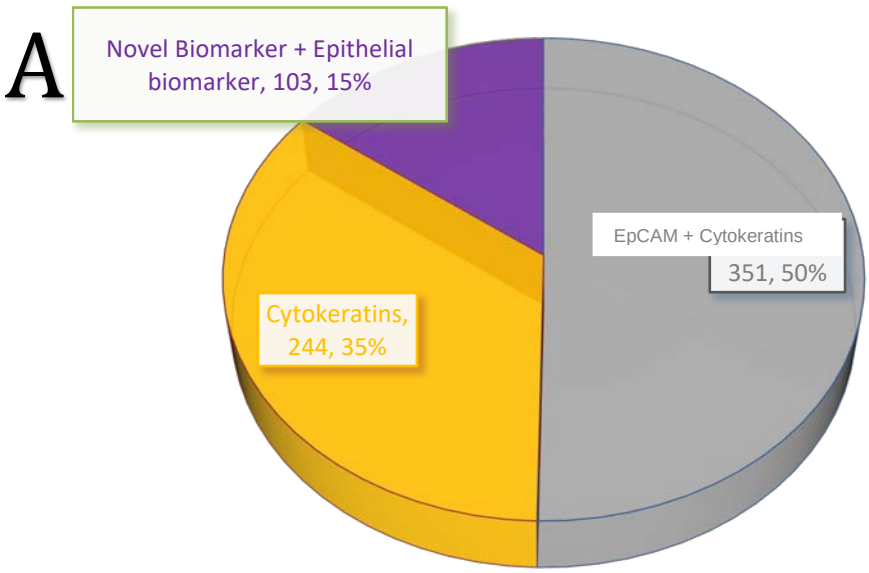
Table 5.2.3.3 –The expression of novel biomarkers in CTCs in patients with Stage I and II oesophageal cancer undergoing surgery alone

Patient	Pre-operative blood sample Novel biomarker expression	Total no of CTCs pre-operative sample – Novel biomarkers	Postoperative blood sample Novel biomarker expression	Total no of CTCs postoperative sample – Novel biomarkers
A18	Vimentin = 2	2		
A19	E-cadherin + Vimentin + EpCAM and cytokeratins = 3 Vimentin + EpCAM and cytokeratins = 2 c-Met = 2	7	c-Met + EpCAM = 5 E-cadherin + N-cadherin + EpCAM and cytokeratins = 2 N-cadherin + EpCAM and cytokeratins = 3	10
A20	ERCC1 + cytokeratins= 3	3		
A25	N-cadherin = 17	17		
A29	HER2 + cytokeratins = 11	11		

A38	c-Met + EpCAM and cytokeratins = 5	5		

In six of the nine patients with early oesophageal cancer, CTCs which expressed a wide range of epithelial and mesenchymal biomarkers were detected in the pre-operative blood sample. Majority of the CTCs expressed both a novel biomarker and/or EpCAM and/or cytokeratins. However these cells only accounted for a small proportion of the total CTC population in each sample. Only one patient had CTCs which expressed novel biomarkers in both the pre and postoperative sample. Patient A19 had different population of CTCs which expressed the novel biomarkers in the pre and postoperative sample

Figure 5.2.3.4 illustrates the heterogeneity within the CTCs detected in patients with Stage III and IV oesophageal cancer who underwent surgery alone.



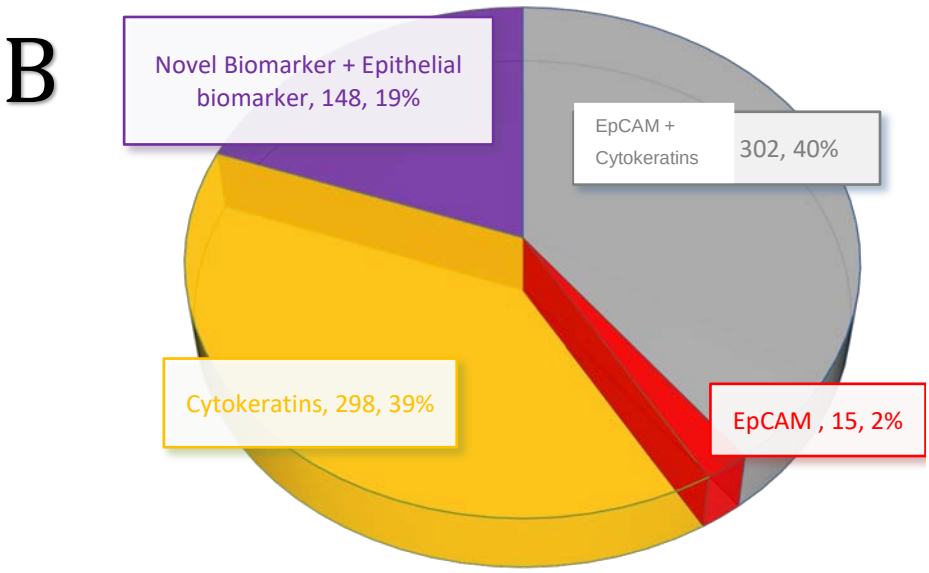


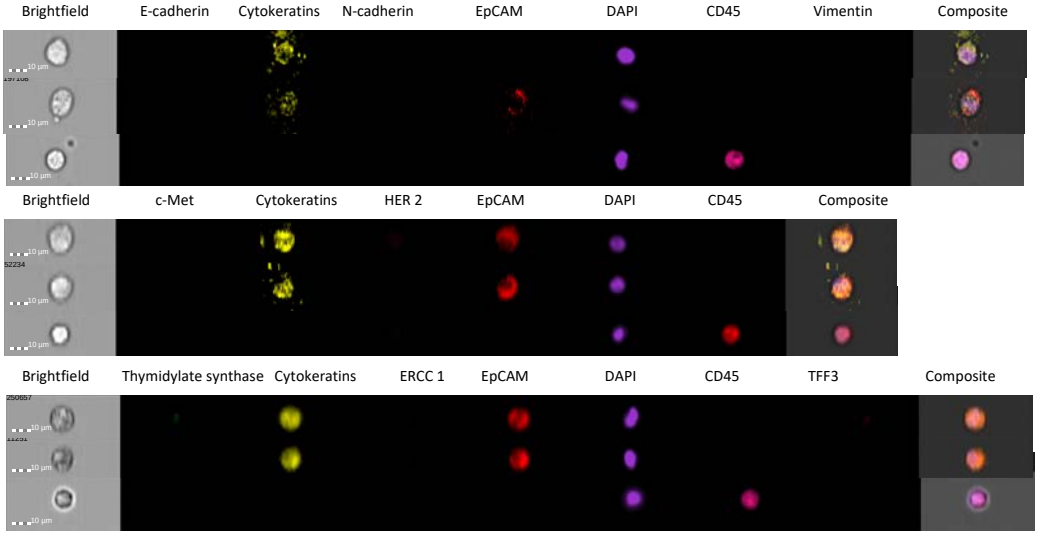
Figure 5.2.3.4 – Heterogeneity of biomarker expression in CTCs detected in patients undergoing surgery alone for Stage III and IV oesophageal cancer who underwent surgery alone.

Biomarker expression in differentiated into five groups: EpCAM and Cytokeratin expression as per CellSearch^{R} selection criteria, EpCAM alone, cytokeratin alone, Novel biomarkers +/- EpCAM +/- cytokeratins– HER2, c-Met, Vimentin, ERCC1, Thymidylate synthase, E-cadherin, N-cadherin, TFF3, Novel biomarkers - HER2, c-Met, Vimentin, ERCC1, Thymidylate synthase, E-cadherin, N-cadherin, TFF3 alone and CTCs which did not express any biomarker. A – CTCs detected in the pre-operative blood sample. B- CTCs detected in the postoperative sample at 48-72 hours.

There were only four patients in this cohort and over 80% of the CTCs detected in this cohort were present in patients A21 and A30. In the pre-operative sample, 50% of the CTCs detected expressed both EpCAM and cytokeratins. Of note, there is marked variation in the biomarker expression in CTCs detected in the pre-operative sample compared to the postoperative samples. The total no of CTCs increased in three out of the four patients in the postoperative sample. However in the postoperative sample only 40% of the CTCs detected expressed both EpCAM and cytokeratins. There was rise in the number of CTCs expressing novel biomarker and an epithelial biomarkers and in CTCs expressing cytokeratins alone. This finding suggests that the source of CTCs in the postoperative sample in the body could be independent of the primary tumour. Thus these CTCs are likely to originate from a distant metastatic site. The population of CTCs expressing novel biomarkers rose between the pre and postoperative sample, 15% to 19%. Figure 5.3.5 illustrates images of CTCs detected in patient A21.

A21 – T3N3 – Surgery alone

CTCs in pre-operative sample



CTCs in postoperative sample

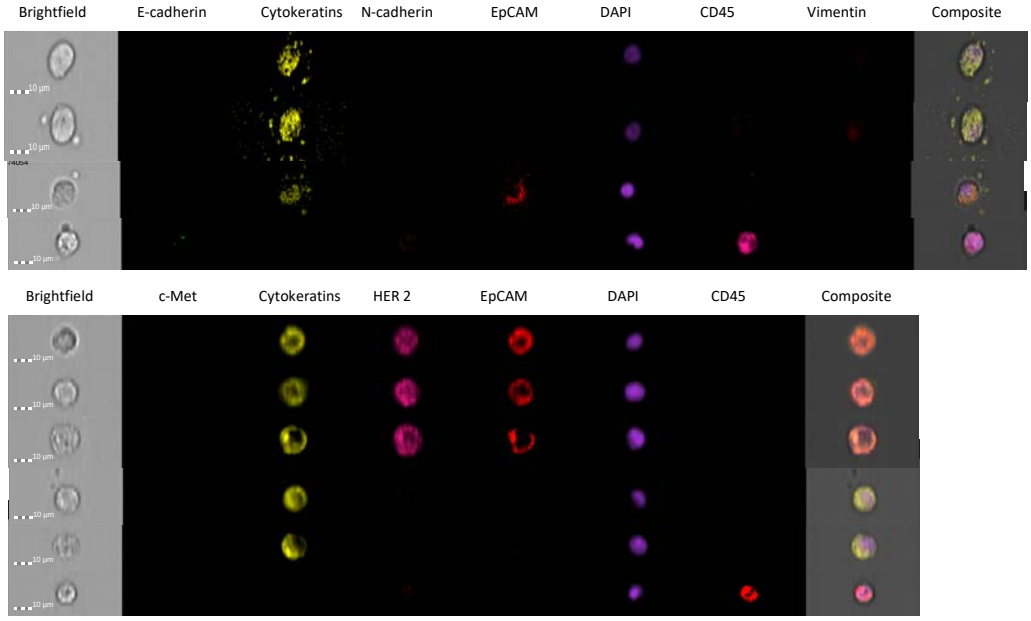


Figure 5.2.3.5 – Images of CTCs detect in Patient A21 who underwent an open oesophagectomy for Stage III oesophageal cancer.

A – Images of CTCs detected in the pre-operative blood sample. B – Images of CTCs detected in the postoperative blood sample at 48 hours

In Patient A21, two populations of CTCs were detected in the pre-operative sample. One population expressed cytokeratins and the other expressed EpCAM and cytokeratins. However, in the postoperative sample, there were two different population of CTCs identified. One population of CTCs expressed cytokeratins and the other populations consisted of CTCs that expressed EpCAM, cytokeratins and HER2.

Figure 5.2.3.6 illustrates the heterogeneity of the novel biomarkers present in the CTCs within this cohort in the pre and postoperative sample.

Figure 5.2.3.6 – The expression of novel biomarkers in CTCs in patients with Stage III and IV oesophageal cancer undergoing surgery alone

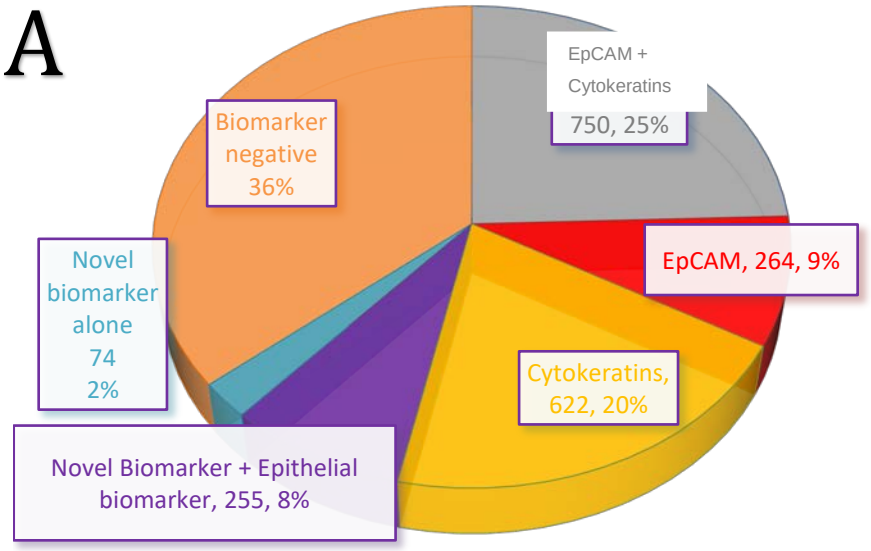
Patient	Pre-operative blood sample Novel biomarker expression	Total no of CTCs/7.5 ml of blood pre-operative sample – Novel biomarkers	Postoperative blood sample Novel biomarker expression	Total no of CTCs /7.5 ml of blood postoperative sample – Novel biomarkers
A7			HER2 + EpCAM = 25	27
A21	Vimentin + cytokeratins = 74	74	HER2 + EpCAM and cytokeratins * = 121	121
A30	Thymidylate synthase + EpCAM and cytokeratins = 23	23		
A36	Vimentin + cytokeratins = 6	6		

*A21 Post op panel C sample lost due to technical reasons

Novel biomarkers were detected in three patients in the pre-operative sample.

Patient A30 had CTCs which expressed thymidylate synthase, EpCAM and cytokeratins. Patient A21 and A36 had CTCs which expressed vimentin and cytokeratins. However, there were no CTCs detected which expressed the novel biomarkers in the postoperative samples in patient A30 and A36. Patients A7 and A21 had CTCs which expressed HER2 in the postoperative sample.

Figure 5.2.3.7 illustrates the heterogeneity within the CTCs detected in patients with Stage III and IV oesophageal cancer who underwent surgery alone.



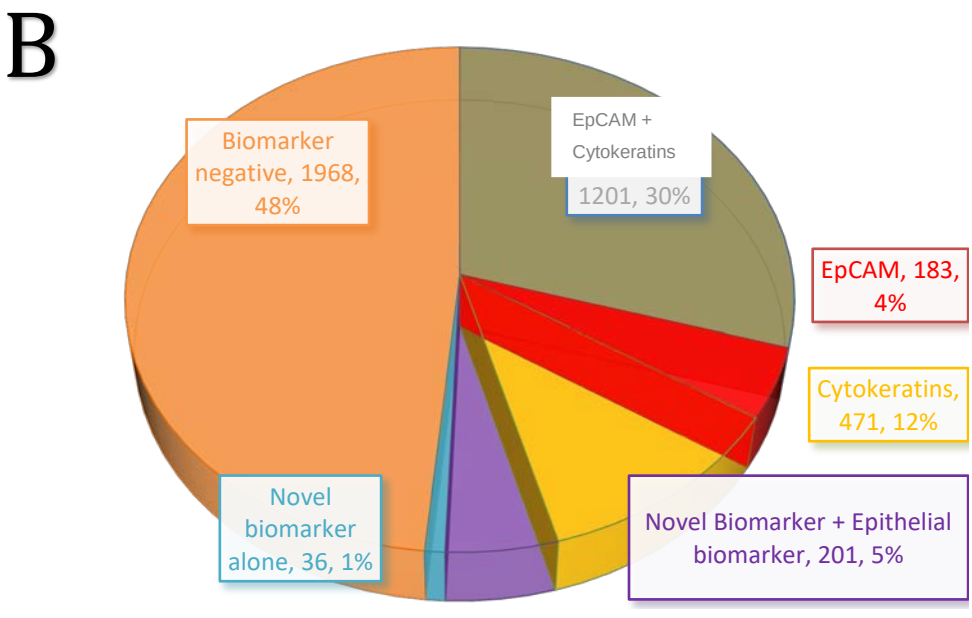


Figure 5.2.3.7 – Heterogeneity of biomarker expression in CTCs detected in patients undergoing surgery alone for Stage III and IV oesophageal cancer who received peri-operative chemotherapy and surgery.

Biomarker expression in differentiated into five groups: EpCAM and Cytokeratin expression as per CellSearch^{R} selection criteria, EpCAM alone, cytokeratin alone, Novel biomarkers +/- EpCAM +/- cytokeratins– HER2, c-Met, Vimentin, ERCC1, Thymidylate synthase, E-cadherin, N-cadherin, TFF3, Novel biomarkers - HER2, c-Met, Vimentin, ERCC1, Thymidylate synthase, E-cadherin, N-cadherin, TFF3 alone and CTCs which did not express any biomarker. A – CTCs per 7.5 ml of blood detected in the pre-operative blood sample. B- CTCs per 7.5 ml of blood detected in the postoperative sample at 48-72 hours.

In this cohort of patients, 36% of the CTCs detected were biomarker negative in the pre and 48% of CTCs in the postoperative samples. This high number could be attributed to the impact of peri-operative chemotherapy in downstaging the tumour. Tumour regression grade indicates response of the primary tumour to the peri-operative chemotherapy. Tumour regression grade three or less indicates a good response to chemotherapy and is a positive prognostic factor. Correlation with the tumour regression grade (TRG) and the number of biomarker negative cells in the pre operative blood sample using Kruskall Wallis test did not reveal a significant difference (p=0.19). However, in the postoperative samples patients with a TRG grade less than 3 had a significantly higher number of biomarker negative cells than those who TRG 4 or greater (p=0.03). This finding suggests that there more biomarker negative cells in patients who have a good response to peri-operative chemotherapy.

The fall in the population of CTCs expressing the novel biomarkers between the pre and postoperative sample was similar to the cohort of patients who had locally advanced cancer who underwent surgery alone. Figure 6.3.9 illustrate images of CTCs present in patient A14 who received peri-operative chemotherapy and surgery.

Figure 5.2.3.8 illustrates the heterogeneity of the novel biomarkers present in the CTCs within this cohort in the pre and postoperative sample

Figure 5.2.3.8 – The expression of novel biomarkers in CTCs in patients with Stage III and IV oesophageal cancer who received peri-operative chemotherapy and surgery.

Patient	Pre-operative blood sample Novel biomarker expression	Total no of CTCs/7.5 ml of blood pre-operative sample – Novel biomarkers	Postoperative blood sample Novel biomarker expression	Total no of CTCs/7.5 ml of blood postoperative sample – Novel biomarkers
A6	No pre-op sample		HER2 = 15	15
A9	ERCC 1 = 37 Vimentin = 6	43	c-Met = 10	10
A10	HER2 + EpCAM = 17	17		
A11	Thymidylate synthase + cytokeratins	14		
A12	Thymidylate synthase + cytokeratins = 3 c-Met + cytokeratins = 26	29	Thymidylate synthase = 7	7
A13	c-Met + cytokeratins = 31	31		
A14	E-cadherin + EpCAM = 38 HER2 + cMet + EpCAM = 35 HER2 + Cytokeratins = 11 Thymidylate synthase + EpCAM= 3	87	E-cadherin + EpCAM = 53 HER2 + EpCAM = 21	74

A16	HER2 + EpCAM and cytokeratins = 32	32	HER2 + EpCAM and cytokeratins = 18	18
A17	Vimentin + EpCAM and cytokeratins = 13	13	Thymidylate synthase + EpCAM and cytokeratins = 8	8
A24	HER2 + EpCAM + cytokeratins = 26	26		
A26	Vimentin = 16	16		
A27	Vimentin = 1	1	Vimentin + EpCAM and cytokeratins = 5	5
A31			c-Met = 3	4
A33			HER2 + EpCAM and cytokeratins = 3	4
A40	No pre-op sample		HER2 + EpCAM and cytokeratins = 85	85

A greater proportion of the patients had CTCs which expressed HER2 were present in this group of patients, four in the pre-operative and five in the postoperative sample. However, in patients A6 and A 40 no pre-operative sample was present in Panel B. Therefore presence of CTCs which expressed HER2 were not detected in the pre-operative sample.

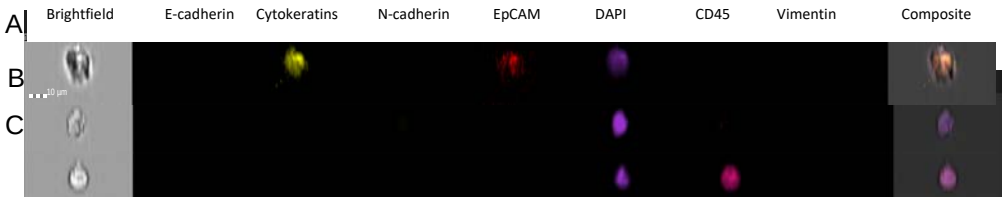
5.2.4 Characterisation of biomarker negative circulating tumour cells in patients undergoing curative resection for oesophagogastric cancer

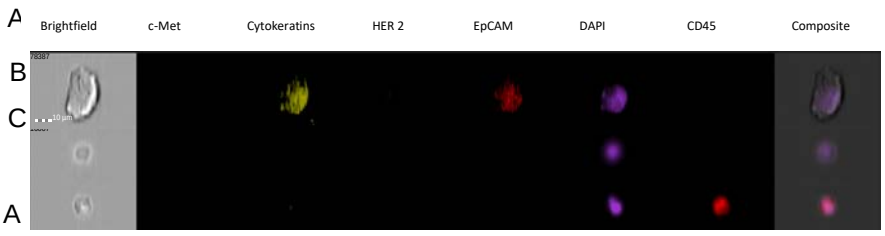
The high resolution imaging flow cytometer allows individual cells to be characterized based on a number of criteria. The size and shape of the cells can be analysed based on their surface area and diameter. An additional benefit of the high resolution imaging flow cytometer is the ability to quantify the intensity of immunofluorescence of individual biomarkers. Hyperploidy within a cell can be analysed and quantified the intensity of the nuclear stain. Quantification of the intensity of immunofluorescence of individual biomarkers further aids in the isolation and identification of CTCs within the blood. The IDEAS software is able to differentiate populations of CTCs based on difference in the intensity of individual biomarkers and the associated immunofluorescence.

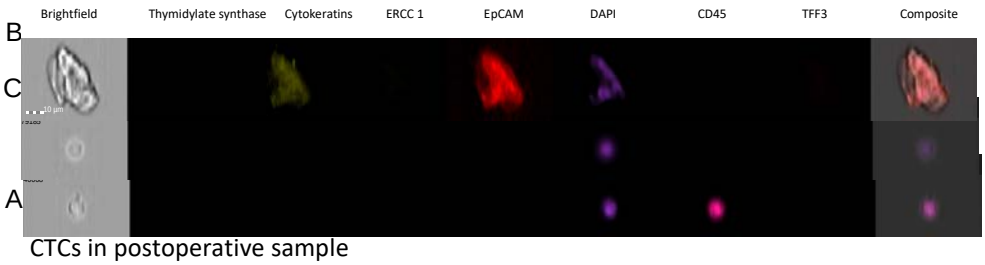
Figure 5.2.4.1 illustrates images of these cells in comparison to white blood cells and CTCs which expressed biomarkers in patients A33.

A33 T3N2 – Peri-operative chemotherapy and Surgery

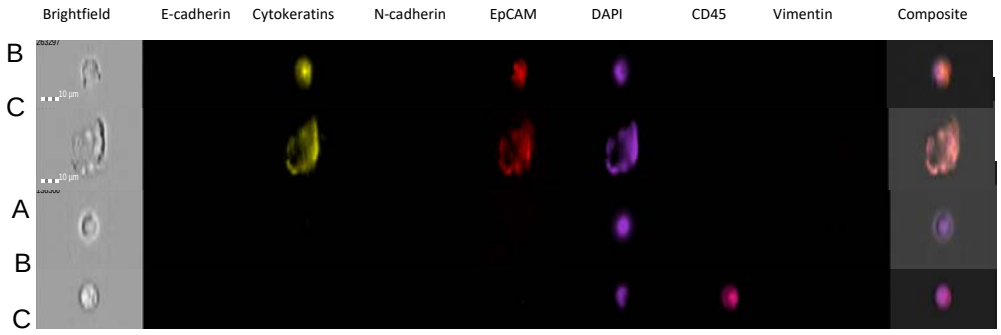
CTCs in pre-operative sample

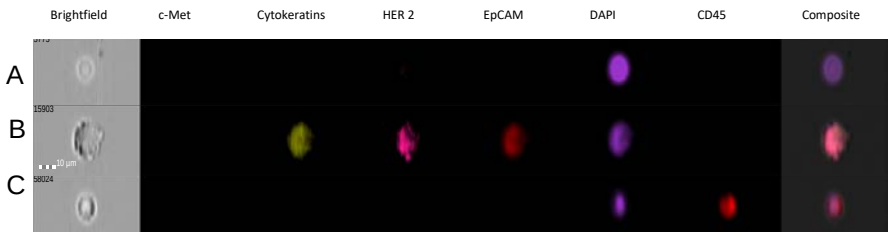






CTCs in postoperative sample





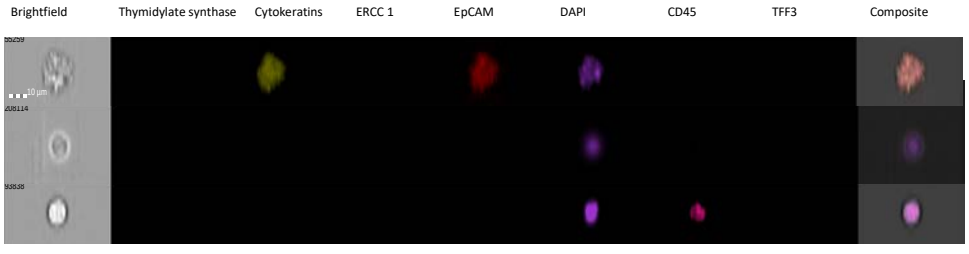


Figure 5.2.4.1 Images of CTCs identified in the pre-operative and postoperative blood sample in patient A33.

A: CTCs which express biomarker, B: CTCs which are biomarker negative C: white blood cells

Figure 5.2.4.2 illustrates the difference in size between the three populations of cells identified in the blood.

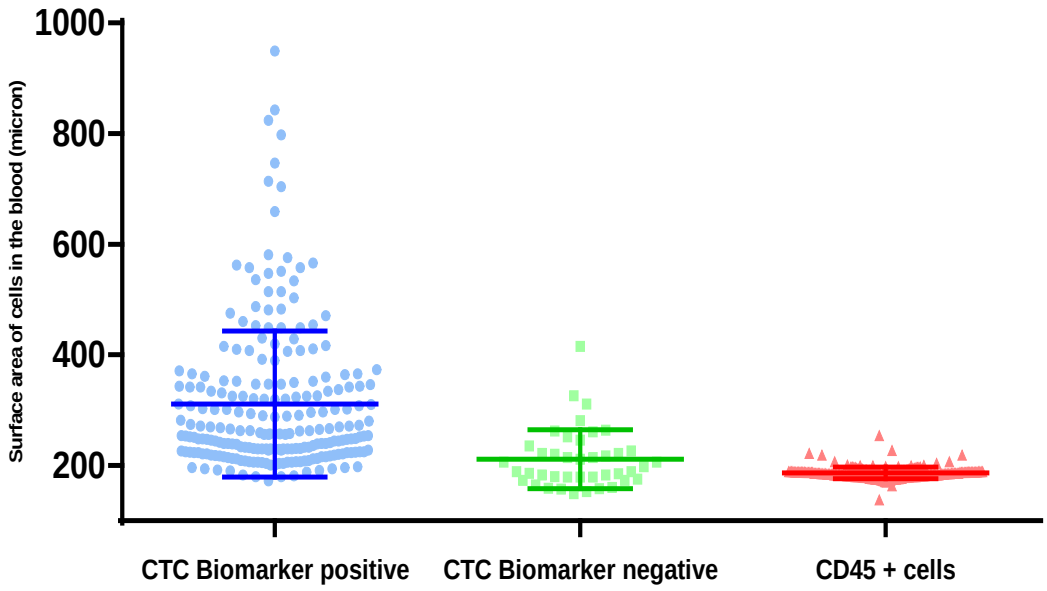


Figure 5.2.4.2 – The surface area of the three populations of cells detected in the blood in patients undergoing curative treatment of oesophageal cancer.

The mean surface area of the CTC which were biomarker positive was 320 μm^2. The mean surface area of CTCs which were biomarker negative was 196 μm^2 and mean surface area of CD45 positive cells was 180 μm^2.

CTCs which expressed a biomarker were significantly larger in size in comparison to white blood cells (p=<0.05) and cells which were biomarker negative (p=<0.05). The cells which were biomarker negative were significantly larger than white blood cells (p= 0.039). The presence of hyperpolidy within a cell can be evaluated by quantifying the intensity of the DAPI nuclear stain. This analysis enables characterisation of CTCs independent of the biomarker expression. Figure 5.2.4.3 illustrates the differences in the DNA nuclear content between the three populations of cells.

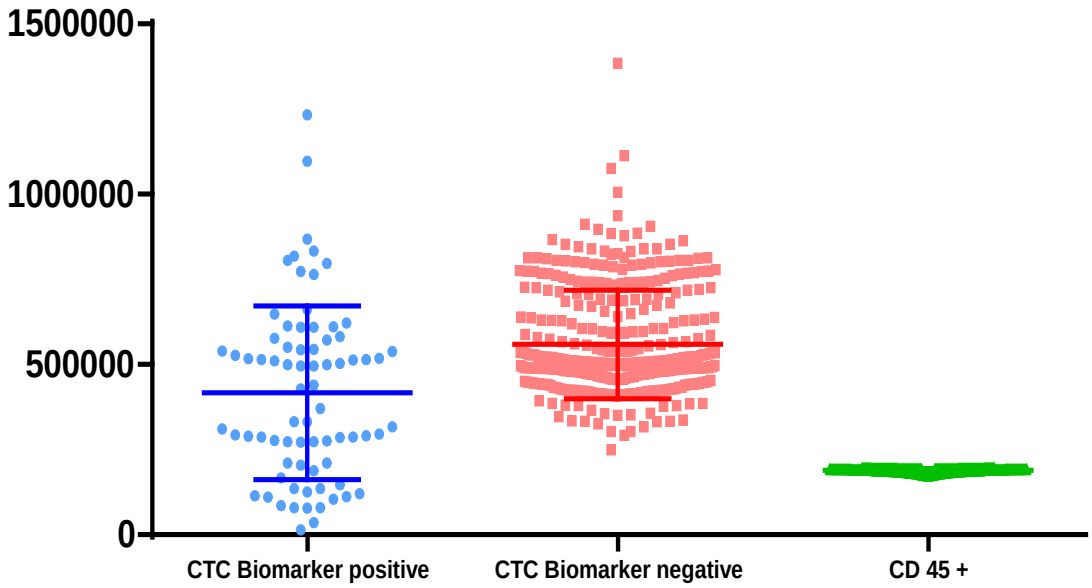


Figure 5.2.4.3– Graph comparing the nuclear density of the three populations of cells detected in the blood in patients undergoing curative treatment of oesophageal cancer.

Biomarker negative cells had a higher nuclear content in comparison to biomarker positive and CD45 + cells.

These analyses enabled the identification of cells within all three cohorts of patients that were morphologically similar to CTCs but did not express biomarkers.

The biomarker negative cells were similar in size to the biomarker positive CTCs but their nuclear density was higher.

Figure 5.2.4.4 illustrates the number of biomarker negative cells identified in the blood in patients undergoing curative treatment for oesophageal adencarcinoma based on the clinical stage of disease.

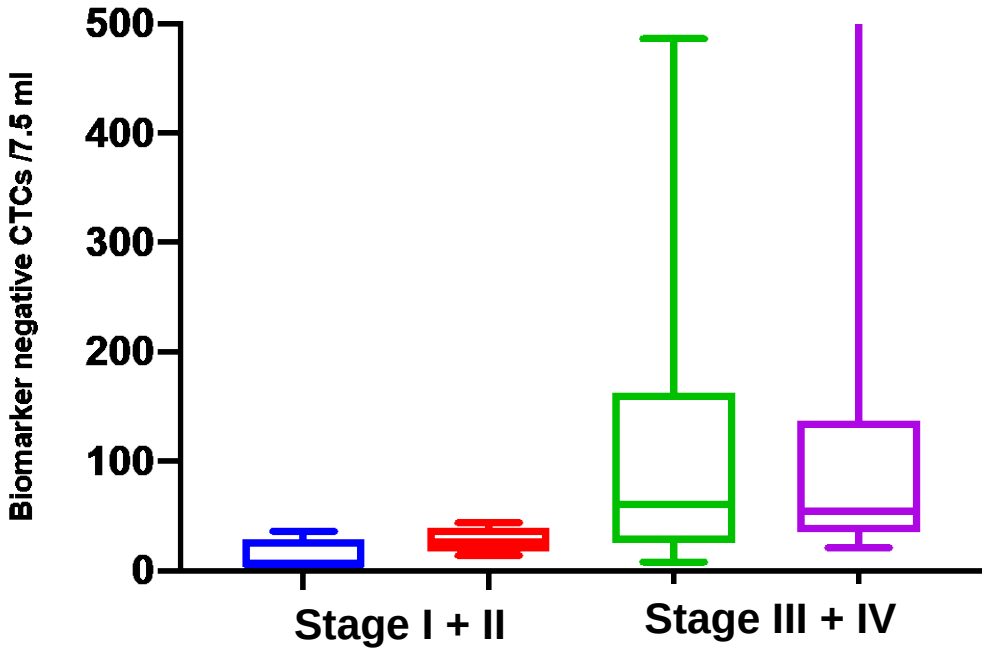


Figure 5.2.4.4 Number of biomarker negative CTCs in 7.5 ml of blood detected in the pre and postoperative sample in patients undergoing curative treatment for oesophageal adenocarcinoma based on the clinical stage of the tumour

In the early cohort, biomarker negative CTCs were detected in the pre-operative sample in 44% (4/9) patients and in the postoperative sample in 44% (4/9) patients. There was a higher proportion of patients with biomarker negative CTCs in the postoperative sample than in the pre-operative sample with Stage III and IV cancer who received peri-operative chemotherapy and surgery, 50% (12/24) versus 38% (9/24). The total number of biomarker negative CTCs detected was higher in the postoperative sample. However, majority of the biomarker negative CTCs detected in the postoperative sample were present in Patient A33 who had 486 biomarker negative CTCs per 7.5 ml of blood in the pre-operative sample, which accounted for 54% of the CTCs detected. In the postoperative sample, there 1035 biomarker negative CTCs per 7.5 ml of blood which accounted for 53% CTCs detected. In the 7 out of the 8 remaining patients, the number of biomarker negative CTCs decreased between the pre and postoperative sample.

There was a variation in the number of biomarker negative CTCs detected in the three cohorts of patients in the study. There was only one patient with Stage III and IV cancer who

underwent surgery alone who had biomarker negative CTCs. Patients with Stage III and IV disease who received per-operative chemotherapy had a higher proportion of biomarker negative CTCs. Biomarker negative CTCs were present in 10 patients (48%) with locally advanced cancer who received peri-oeprative chemotherapy and surgery.

Previous studies have identified CTCs in patients with oesophageal adenocarcinoma based on the postive expression of epithelial biomarkers. The presence of biomarker negative cells has not been previously described before in patients with oesophageal adenocarcinoma. The cells could represent CTCs undergoing EMT transition and not express the epithlial and mesenchymal biomarkers in the study. Further characterisation of these cells could be identified with the detection of transcriptions factors, eg SNAIL1, SNAIL2. In addtion, the next step will be to isolate this population of cells and sequence the genes to identify the molecular origin of the cells.

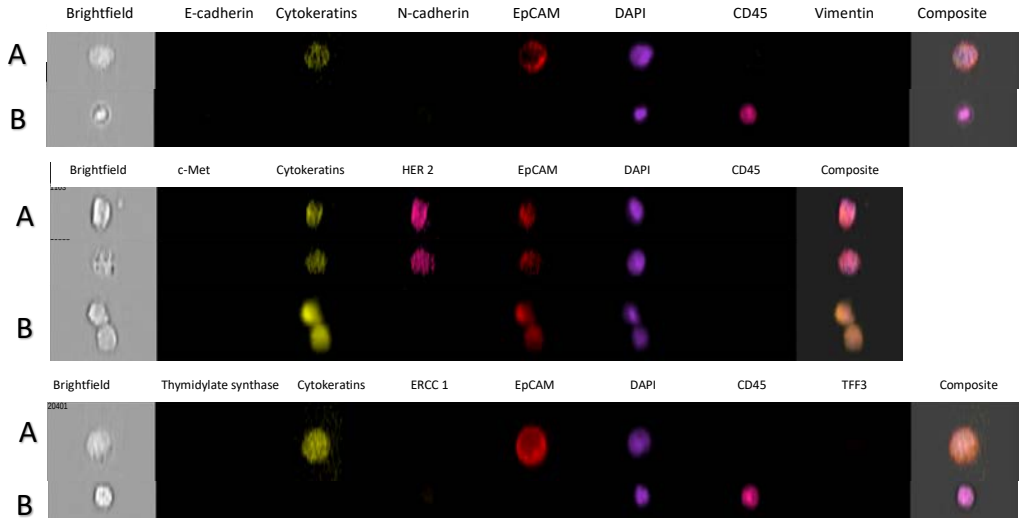
5.2.5 Characterization of disseminated tumour cells detected in patients undergoing curative resection for oesophagogastric cancer

Disseminated tumour cells were isolated from bone marrow of the rib section that is excised as part of an open oesophagectomy. Of the nine patients with early oesophageal cancer, six underwent an open oesophagectomy and bone marrow samples from the rib section was obtained for these patients. The other three patients underwent a thoracoscopic oesophagectomy.

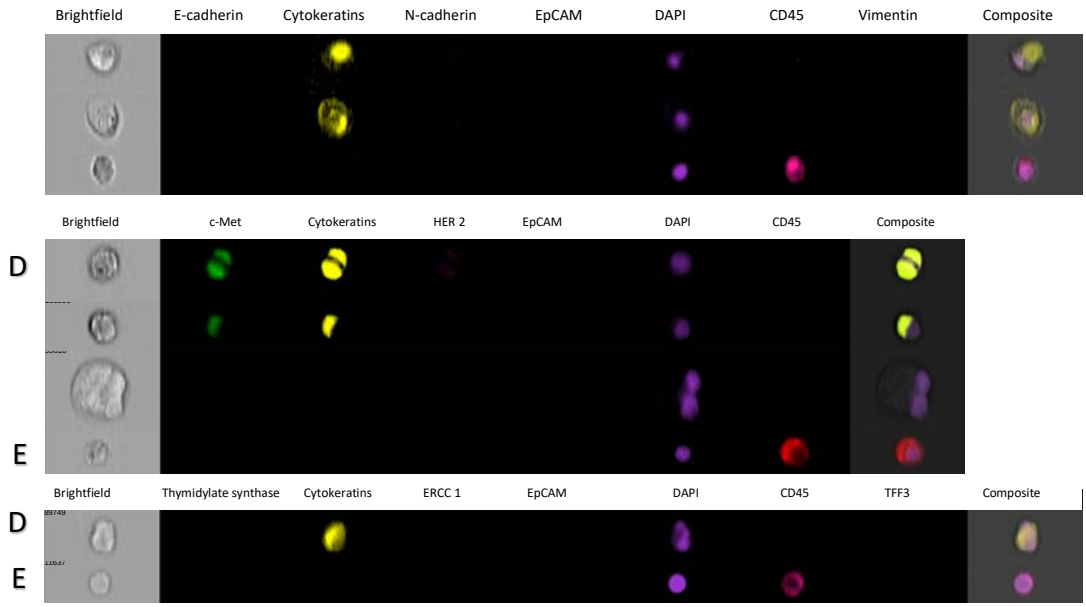
Figure 5.2.5.1 illustrates the images of CTCs identified in the pre and postoperative blood sample and DTCs in the bone marrow in patient A34.

A34 T1bN0 – Surgery alone

CTCs in pre-operative sample



DTCs in bone marrow of excised rib



CTCs in the postoperative sample

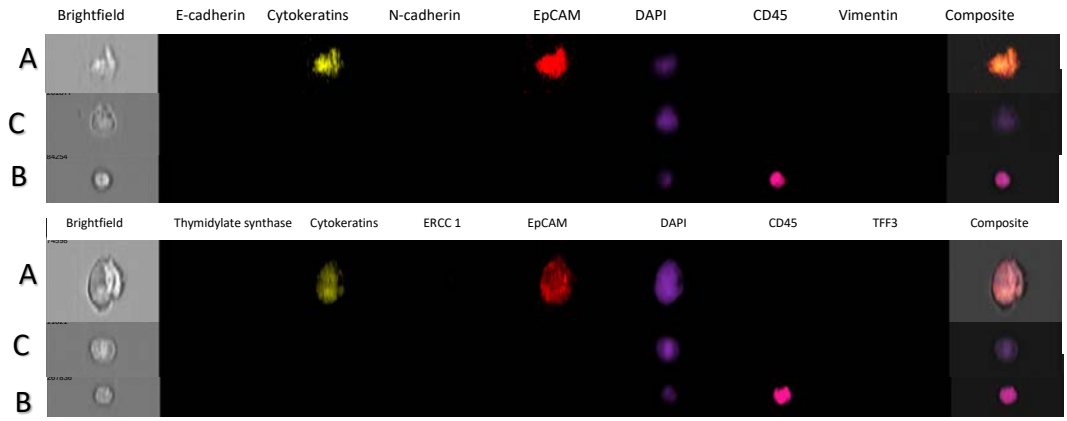


Figure 5.2.5.1– Images of circulating tumour cells in the blood and disseminated tumour cells in the bone marrow of excised rib in patient A34 who underwent an open oesophagectomy for Stage I cancer.

A: Biomarker positive CTCs detected in the pre and postoperative blood sample. B: white blood cells in the pre and postoperative blood sample C: Biomarker negative cells in the pre and postoperative blood sample D: DTCs detected in the bone marrow from the excised rib. E: white blood cells detected in the bone marrow from the excised rib

80/110 CTCs detected in the pre-operative blood sample expressed cytokeratin and EpCAM. Three populations of DTCs identified in the bone marrow. DTCs which expressed c-Met alone, cytokeratins alone and large biomarker negative cells. There were two population of CTCs in the postoperative sample, one population expressed EpCAM and cytokeratins and the other were biomarker negative.

Figure 5.2.5.2 illustrates the presence of two different CTC populations between the pre- and postoperative sample. CTCs detected in the pre-operative sample expressed both EpCAM and cytokeratins and CTCs in the postoperative sample expressed cytokeratin only. DTCs in the bone marrow expressed cMET alone and cytokeratins alone. This could explain the origin of CTCs in the postoperative sample originating from the bone marrow.

Figure 5.2.5.2 illustrates the biomarker expression in the disseminated tumour cells isolated in this cohort of patients.

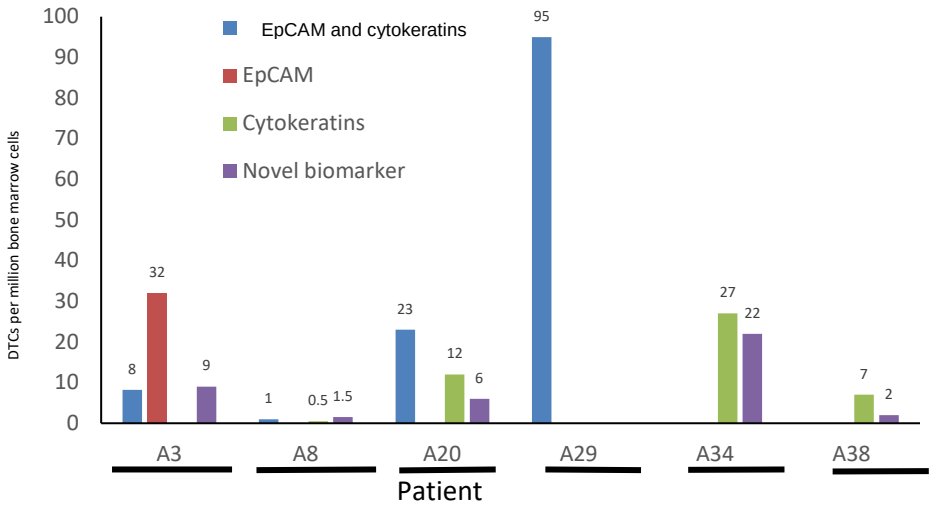
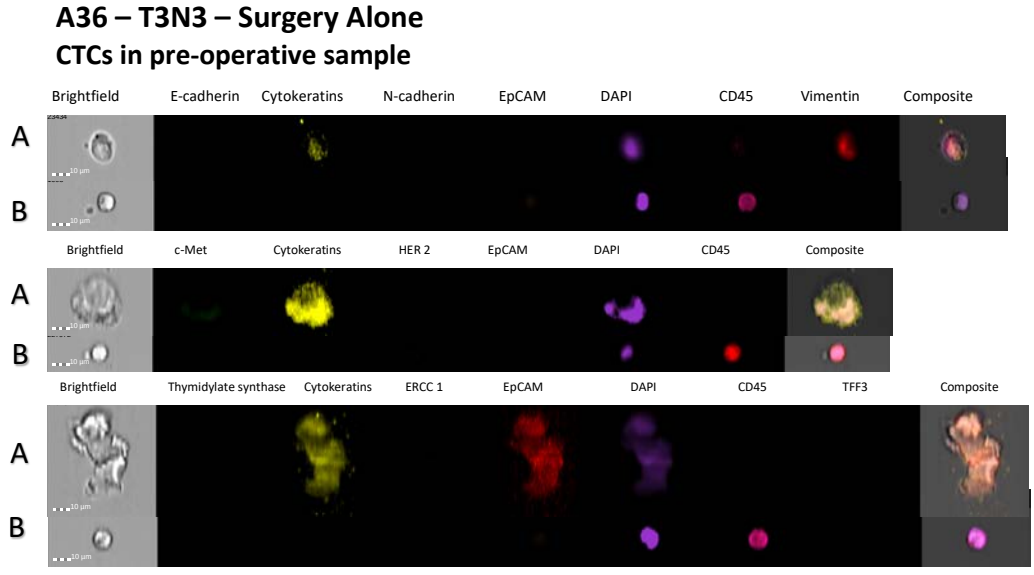


Figure 5.2.5.2– The number of disseminated tumour cells in the bone marrow in patients with Stage I and II cancer who underwent an open oesophagectomy.

A - DTCs that express EpCAM and cytokeratins biomarkers as per the CellSearch^{R} test B: DTCs that express EpCAM, C: DTCs that express cytokeratins, D: DTCs that express novel biomarkers +/- epithelial biomarker - HER2, c-Met, E-cadherin, N-cadherin, Thymidylate synthase

Patients A3, A20 and A34 had a higher number of DTCs in comparison to the other patients in the cohort. Patient A34, there were two populations of DTCs identified. Patient A3, had three populations of DTCs. The first population consisted of DTCs expressed EpCAM alone, the second population of DTCs expressed EpCAM and HER2 and the third population expressed EpCAM and cytokeratin.

All four patients with clinical stage III and IV cancer underwent open oesophagectomy as the as their mainstay of curative treatment. Figure 5.2.5.3 illustrates images of CTCs and DTCs detected in patient A36 who underwent an open oesophagectomy for locally advanced oesophageal cancer.



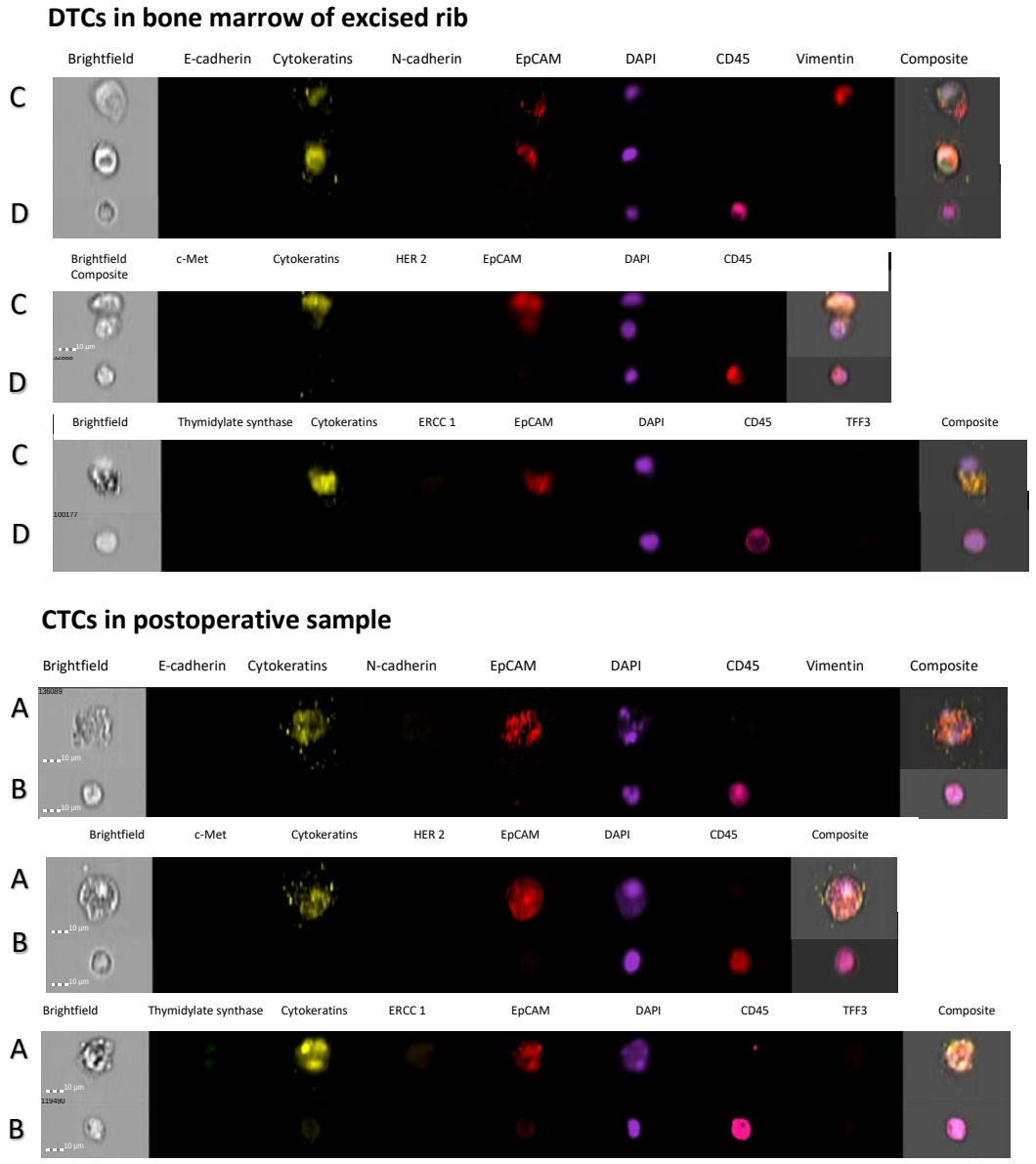


Figure 5.2.5.3– Images of circulating tumour cells in the blood and disseminated tumour cells in the bone marrow of excised rib in patient A36 who underwent an open oesophagectomy for Stage III cancer.

A: CTCs which are biomarker positive B: white blood cells in the blood. C: DTCs detected in the bone marrow which are biomarker positive, D: white blood cells in the bone marrow.

CTCs in the pre-operative blood samples expressed cytokeratins alone and in the postoperative sample expressed EpCAM and cytokeratins. There were two population of DTCs present, one population expressed EpCAM and cytokeratins and the other expressed EpCAM, cytokeratins and Vimentin. There are two different populations of DTCs present in the bone marrow in patient A36. The biomarker expression in the postoperative CTCs is similar to the DTCs detected

in the bone marrow. Of interest, only one population of CTCs were detected in the postoperative sample which expressed EpCAM and cytokeratins.

Figure 5.2.5.4 illustrates the number of disseminated tumour cells identified in each patient.

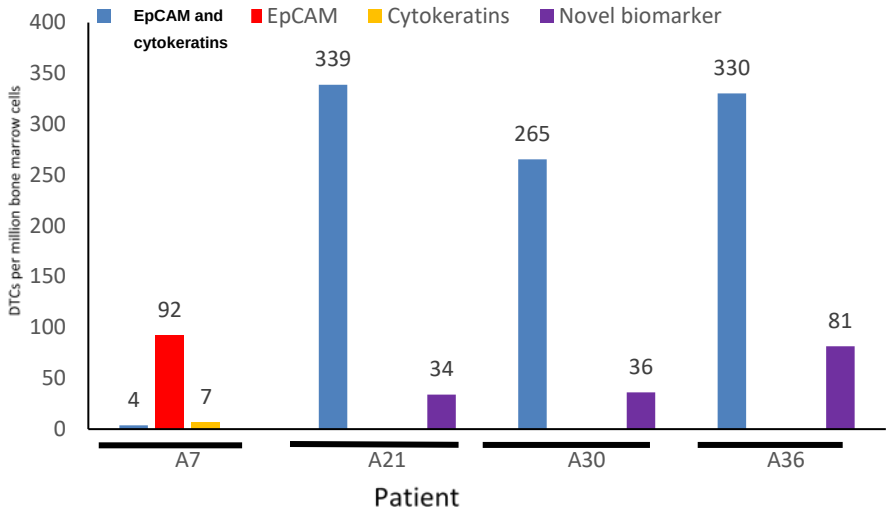


Figure 5.2.5.4– The number of disseminated tumour cells in the bone marrow in patients with Stage III and IV cancer who underwent an open oesophagectomy.

A - DTCs that express EpCAM and cytokeratins biomarkers as per the CellSearch^{R} test B: DTCs that express EpCAM, C: DTCs that express cytokeratins, D: DTCs that express novel biomarkers +/- epithelial biomarker - HER2, c-Met, E-cadherin, N-cadherin, Thymidylate synthase

There was no significant difference in the number of biomarker positive DTCs in the bone marrow between patients with early and locally advanced oesophageal cancer who underwent surgery alone, comparison with Mann Whitney test p=0.992. The majority of DTCs detected in this cohort expressed both EpCAM and cytokeratins. DTCs which expressed cytokeratins alone were not detected.

Twenty-four patients with Stage III and IV oesophageal cancer underwent an open oesophagectomy following peri-operative chemotherapy. Figure 5.2.5.5 illustrates the number of disseminated tumour cells detected in this cohort of patients.

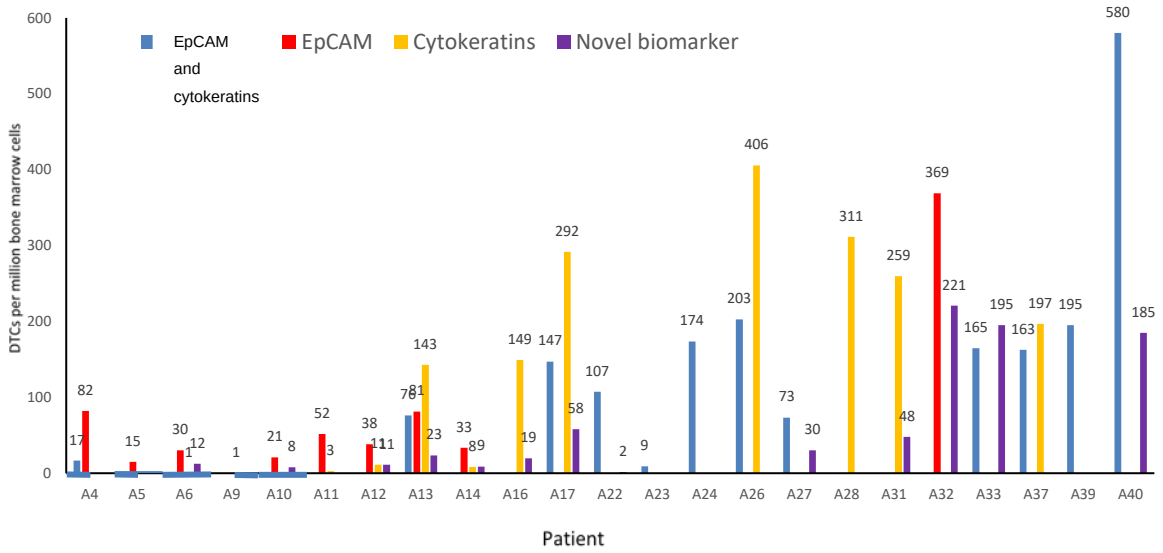
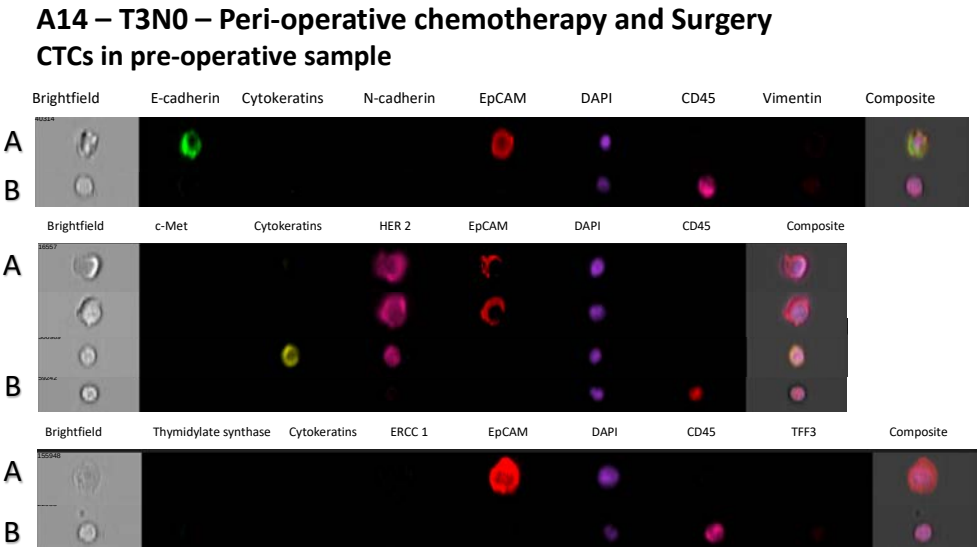


Figure 5.2.5.5– The number of disseminated tumour cells in the bone marrow in patients with Stage III and IV cancer who recrieved peri-operative chemotherapy and underwent an open oesophagectomy.

A - DTCs that express EpCAM and cytokeratins biomarkers as per the CellSearch^{R} test B: DTCs that express EpCAM, C: DTCs that express cytokeratins, D: DTCs that express novel biomarkers +/- epithelial biomarker - HER2, c-Met, E-cadherin, N-cadherin, Thymidylate synthase

There was a wide variation in the number of DTCs detected in this cohort of patients. In a proportion of the patients the number of DTCs detected was similar to patients with early oesophageal cancer. Figure 5.2.5.6 illustrates images of CTCs and DTCs detected in patient A14.



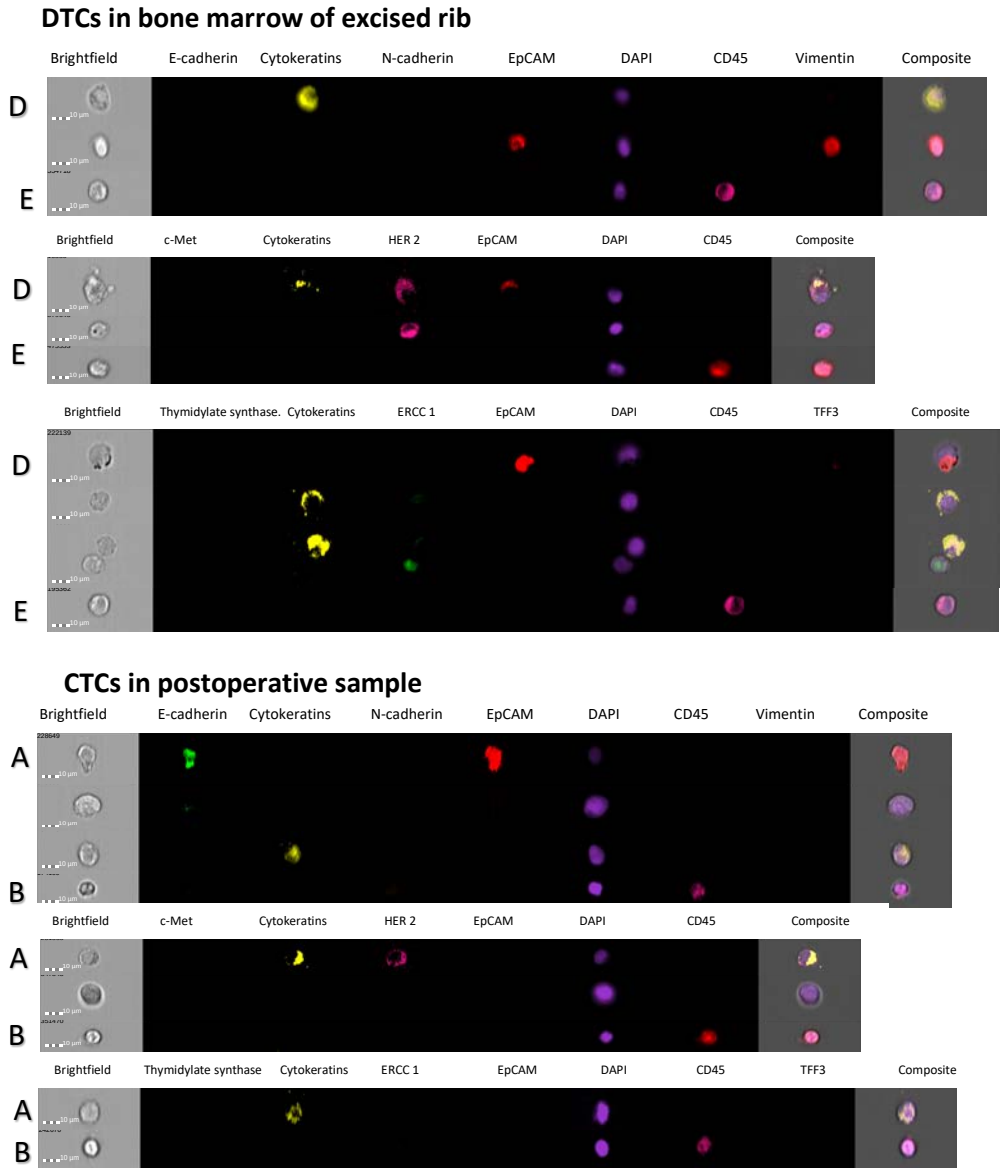


Figure 5.2.5.6– Images of circulating tumour cells in the blood and disseminated tumour cells in the bone marrow of excised rib in patient A14 who received peri-operative chemotherapy and underwent an open oesophagectomy for Stage III cancer.

A: Biomarker positive CTCs detected in the pre and postoperative blood sample. B: white blood cells in the pre and postoperative blood sample C: Biomarker negative cells in the pre and postoperative blood sample D: DTCs detected in the bone marrow from the excised rib. E: white blood cells detected in the bone marrow from the excised rib

Four populations of CTCs were detected in the three panels in the pre-operative sample: CTCs which expressed EpCAM alone, CTCs which expressed EpCAM and HER2, CTCs which

expressed E-cadherin and EpCAM and CTCs which expressed cytokeratins and HER2. This sample highlights the heterogeneity of biomarker expression present in the CTCs in the blood. The presence of different biomarkers in the three panels aids in the characterization of the heterogeneity. It is possible that the CTCs which express EpCAM in panel C are part of the same population present in panel B in which they express EpCAM and HER2 and in the panel A in which they expressed E-cadherin and EpCAM.

In the bone marrow, there were five different population of DTCs present: DTCs which expressed EpCAM alone, DTCs which expressed cytokeratins alone, DTCs which expressed HER2 alone, DTCs which expressed EpCAM, cytokeratins and HER2 and DTCs which expressed ERCC1 alone.

In the postoperative sample, there were four different populations of CTCs present: CTCs which expressed cytokeratins and HER2, CTCs which expressed cytokeratins alone, CTCs which expressed E-cadherin and EpCAM and CTCs which were biomarker negative.

The preceding data suggests that the potential origin of the CTCs in the postoperative sample is from the bone marrow. Further correlations of the biomarker expression in DTCs and CTCs in the pre and postoperative blood sample are noted in Tables 5.2.5.7, 5.2.5.8 and 5.2.5.9.

Table 5.2.5.7: Table comparing the biomarkers expression of DTCs in the bone marrow in the rib and CTCs in the pre and postoperative sample in patients underwent an open oesophagectomy for Stage I and II oesophageal cancer

Patient Identifier	Biomarker Expression Pre-op CTC	Biomarker Expression DTC	Biomarker Expression Post-op CTC
A3	EpCAM	EpCAM and cytokeratins EpCAM HER2	EpCAM
A8	Cytokeratins	EpCAM and cytokeratins Cytokeratins ERCC 1	

	CD 45 -		CD 45 -
A20	EpCAM and cytokeratins Cytokeratins ERCC 1 + Cytokeratins	EpCAM and cytokeratins Cytokeratins E Cadherin	EpCAM and cytokeratins Cytokeratins
A29	 Cytokeratins HER2 + Cytokeratins	EpCAM	 Cytokeratins
A34	EpCAM and cytokeratins	 Cytokeratins cMET + Cytokeratins Vimentin + Cytokeratins	EpCAM and cytokeratins CD 45 -
A38	 Cytokeratins cMET	 Cytokeratins HER2 + Cytokeratins	EpCAM and cytokeratins CD 45 -

There was evidence of two or more different population of DTCs present in the bone marrow based on biomarker expression in this group of patients. In three out of the six patients, the biomarker expression in the pre and post-operative CTCs were similar. Patients A34 and A38 had CTCs in the pre-operative sample which expressed two or more different biomarkers when compared to the post-operative CTCs which expressed only one biomarker. In two patients, A3 and A20 the CTCs in the post-operative sample expressed the same biomarkers as those present in the DTCs in the bone marrow.

Table 5.2.5.8 illustrates the biomarker expression in DTCs in the bone marrow and CTCs in the pre and post-operative sample in patients who underwent an open oesophagectomy for Stage III and IV cancer.

Table 5.2.5.8: Table comparing the biomarkers expression of DTCs in the bone marrow in the rib and CTCs in the pre and postoperative sample in patients underwent an open oesophagectomy for Stage III and IV oesophageal cancer

Patient Identifier	Biomarker Expression Pre-op CTC	Biomarker Expression DTC	Biomarker Expression Post-op CTC
A7	EpCAM and cytokeratins	EpCAM and cytokeratins EpCAM Cytokeratins	EpCAM and cytokeratins EpCAM Cytokeratins HER2 + EpCAM
A21	EpCAM and cytokeratins Vimentin + Cytokeratins	EpCAM and cytokeratins HER2 + Cytokeratins	EpCAM and cytokeratins Cytokeratins HER2 + EpCAM and cytokeratins
A30	EpCAM and cytokeratins Cytokeratins Thymidylate synthase + EpCAM + Cytokeratins	EpCAM and cytokeratins HER2	EpCAM and cytokeratins Cytokeratins
A36	Cytokeratins Vimentin + Cytokeratins	EpCAM and cytokeratins Vimentin + Cytokeratins	EpCAM and cytokeratins

In three out of the four patients in this cohort, the biomarker expression was similar between the DTCs in the bone marrow and CTCs in the pre and post-operative sample. Furthermore, the biomarker expression of at least one population of CTCs in the post-operative sample and DTCs was the same in all four patients. In patients A7, A20 and A36, CTCs which expressed EpCAM and cytokeratins were present in the pre and post-operative sample and in the DTCs in the bone marrow.

Table 5.2.5.9 illustrates the biomarker expression in DTCs in the bone marrow and CTCs in the pre and post-operative sample in patients who received peri-operative chemotherapy and underwent an open oesophagectomy for Stage III and IV cancer.

Table 5.2.5.9: Table comparing the biomarkers expression of DTCs in the bone marrow in the rib and CTCs in the pre and postoperative sample in patients who received peri-operative

Patient Identifier	Biomarker Expression Pre-op CTC	Biomarker Expression DTC	Biomarker Expression Post-op CTC
A4	EpCAM	EpCAM and cytokeratins EpCAM	EpCAM
A5	EpCAM and cytokeratins	EpCAM Cytokeratins Vimentin	Cytokeratins
A6	No sample	EpCAM and cytokeratins EpCAM Cytokeratins HER2, Vimentin	EpCAM Cytokeratins HER2
A9		EpCAM Cytokeratins	EpCAM

	ERCC 1, Vimentin CD 45 -	TFF3	cMET CD 45 -
A10	 HER2 + EpCAM CD 45 -	Cytokeratins HER2 + EpCAM	 CD 45 -
A11	 Thymidylate synthase + Cytokeratins CD 45 -	 EpCAM Cytokeratins	EpCAM and cytokeratins EpCAM Cytokeratins CD 45 -
A12	EpCAM and cytokeratins Cytokeratins Thymidylate synthase + Cytokeratins cMET+ Cytokeratins CD 45 -	 EpCAM Cytokeratins Survivin	 EpCAM Cytokeratins Thymidylate synthase CD 45 -
A13	 Cytokeratins	EpCAM and cytokeratins EpCAM Cytokeratins	 Cytokeratins

	cMET + Cytokeratins	Thymidylate synthase	
A14	EpCAM HER2 + Cytokeratins E Cadherin + EpCAM HER2 + EpCAM	EpCAM Cytokeratins HER2 + Cytokeratins ERCC1	 Cytokeratins HER2 + Cytokeratins E Cadherin + EpCAM CD 45 -
A16	EpCAM and cytokeratins HER2 + EpCAM and cytokeratins	 Cytokeratins Vimentin	EpCAM and cytokeratins Cytokeratins HER2 + EpCAM and cytokeratins
A17	EpCAM and cytokeratins Vimentin + EpCAM and cytokeratins	EpCAM and cytokeratins Cytokeratins Vimentin	EpCAM and cytokeratins Cytokeratins Vimentin, TFF 3
A22	 CD 45 -	EpCAM and cytokeratins HER2	EpCAM and cytokeratins Thymidylate synthase + EpCAM and cytokeratins CD 45 -
A23	 Cytokeratins	EpCAM and cytokeratins	EpCAM and cytokeratins CD 45 -

A24	EpCAM and cytokeratins HER2 + EpCAM and cytokeratins CD 45 -	EpCAM and cytokeratins	EpCAM and cytokeratins CD 45 -
A26	Cytokeratins Vimentin CD 45 -	EpCAM and cytokeratins Cytokeratins	EpCAM CD 45 -
A27	EpCAM Vimentin	EpCAM and cytokeratins Vimentin + EpCAM and cytokeratins	EpCAM and cytokeratins Cytokeratins Vimentin + EpCAM and cytokeratins CD 45 -
A28	EpCAM and cytokeratins EpCAM	Cytokeratins	EpCAM and cytokeratins Cytokeratins
A31	EpCAM and cytokeratins Cytokeratins CD 45 -	Cytokeratins Thymidylate synthase	EpCAM and cytokeratins c-Met
A32	EpCAM and cytokeratins		EpCAM and cytokeratins

	CD 45 -	EpCAM Thymidylate synthase	
A33	EpCAM and cytokeratins CD 45 -	EpCAM and cytokeratins Thymidylate synthase, HER2 + EpCAM Vimentin	EpCAM and cytokeratins HER2 + EpCAM CD 45 -
A37	EpCAM	EpCAM and cytokeratins Cytokeratins	EpCAM and cytokeratins
A39	EpCAM and cytokeratins Cytokeratins	EpCAM and cytokeratins	EpCAM and cytokeratins Cytokeratins CD 45 -
A40	EpCAM and cytokeratins Cytokeratins No panel B	EpCAM and cytokeratins Thymidylate synthase	EpCAM and cytokeratins HER2 + EpCAM

*A35 excluded - metastatic disease at time of surgery and no resection was performed

As described in the previous cohort of patients, there were multiple populations of DTCs present in the bone marrow and CTCs in the blood. In twenty out of twenty-three patients there was at least one population of CTCs in the postoperative sample with the same biomarker expression as a population of DTCs in the bone marrow. In four patients in this cohort, the expression of the novel biomarkers (HER2, vimentin, thymidylate synthase) was

similar between the DTCs and the CTCs in the postoperative sample. In thirteen patients, the biomarker expression of the CTCs of at least one population of cells in the pre-operative sample were similar to the cells present in the postoperative sample. However, only in 9 out of the 13 patients, there was at least one population of DTCs present in the bone marrow which expressed the same biomarkers as those present in the pre and postoperative sample. In most of these patients, only the epithelial biomarkers (EpCAM, cytokeratins) remain similar between the CTCs in the pre and postoperative blood sample and the DTCs in the bone marrow. There is variation in the expression of the novel biomarkers between the three sites. There were biomarker negative cells in the postoperative sample in 12 out of the twenty three patients in comparison to only 9 out of twenty three patients in the pre-operative sample. In 8 patients, biomarker negative cells were present in both the pre and post-operative sample.

Kruskall Wallis analysis was performed to determine the statistical correlation of biomarker expression. There was no significant correlation in the biomarker expression between the pre-operative CTCs, DTCs in the bone marrow and postoperative CTCs in patients undergoing treatment for early cancer ($p=0.470$) and locally advanced cancer who received peri-operative chemotherapy and surgery ($p=0.655$). There was a significant correlation in patients with locally advanced cancer who underwent surgery alone ($p=<0.05$). This could indicate that the CTCs in these patients are originating from the bone marrow rather than the primary tumour and thus the population of CTCs remain in the postoperative sample following resection of the primary tumour. Definitive conclusions are difficult due to the small cohort of patients.

Further analysis of interest would be to compare the biomarker expression in the primary tumour and lymph nodes to the CTCs in the blood sample and the DTCs in the bone marrow. This analysis may aid in identifying the origin of the CTCs present in the circulation. In addition, sequencing the individual populations of CTCs and DTCs present after selection based on their biomarker expression would help understand the origin of these cells.

5.2.6 Characterisation of the morphology and biomarker expression of disseminated tumour cells in the bone marrow in patients undergoing curative resection for oesophagogastric cancer

Two population of cells were identified within the bone marrow that from samples obtained from patients undergoing elective orthopaedic surgery. The first population consisted of cells which expressed the CD45 biomarker. The second population consisted of cells which were biomarker negative and smaller in size than the CD45 positive cells. Both these cell populations were present in the bone marrow from the rib section resected in all the cancer patients in the study. Figure 6.2.6.1 illustrates the difference in surface area of the three population of cells identified in the bone marrow based on biomarker expression.

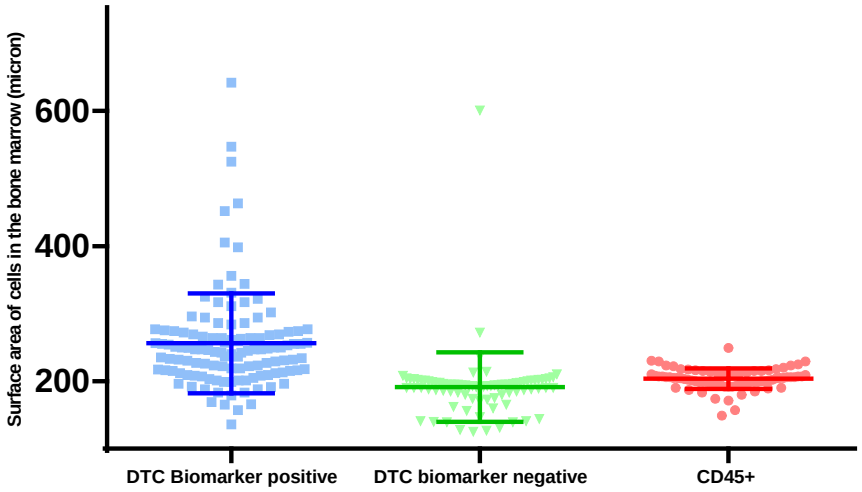


Figure 5.2.6.1– The surface areas of the three populations of cells detected in the bone marrow in patients undergoing curative treatment of oesophageal cancer

The three populations of cells were identified in the bone marrow obtained from the rib. The first population consisted of cells which expressed either one or more of the biomarkers present in the panel and classified as DTCs. The second population consisted of cells which were biomarker negative and the third population consisted of cells which expressed the CD45 biomarker.

The majority of the DTCs were larger in size than the cells which expressed CD 45 biomarker and the cells which were biomarker negative (p=0.244). Furthermore, there was a wide variation in the surface area of the DTCs present in the bone marrow.

5.2.7 Association between oncological outcomes and the presence of circulating and disseminated tumour cells in patients undergoing curative treatment for oesophagogastric cancer

Prognostic factors which determine survival following curative surgery are determined by the pathological findings on the resected specimen. They are a measure of the success of the curative treatment that the patient has received. There are surgical guidelines that determine the quality of the surgery based on two pathological findings (155). Patients undergoing curative resection should have a minimum of 15 lymph nodes excised to stage accurately the tumour and a R0 resection status should be achieved which indicates that the resection margins are clear of tumour invasion. Standardized operative technique combined with radical lympadenectomy achieves this goal. The pathologist grades the specimen on the following criteria: pathological stage of the tumour, lymph node yield, resection margin, lymphovascular invasion (LVI) and tumour regression grade in patients who have received peri-operative chemotherapy. Each of these criteria are independent prognostic markers of survival in patients undergoing curative treatment for oesophagogastric cancer.

In the early cancer cohort, there were nine patients who were staged clinically with $\leq$ T2 disease with no evidence of nodal metastases at the time of surgery. Post resection, one patient had evidence of nodal metastases. This patient would have been considered for peri-operative chemotherapy at the time of surgery if the nodal metastases had been identified at the time of clinical staging. In the cohort of patients who had locally advanced cancer who underwent surgery alone, all four patients had evidence of nodal disease following resection. Nodal metastases had been identified in two of these patients at the time of clinical staging. These three cases highlight the challenges in identifying nodal metastases with the current staging modalities. The median lymph node yield in the study was 33 (24-105). A R0 resection margin was achieved in all patients undergoing curative resection. Figure 5.2.7.1 and Figure 5.2.7.2 illustrate the correlation between the number of biomarker positive circulating tumour cells detected in patients and the clinical and pathological stage of the disease.

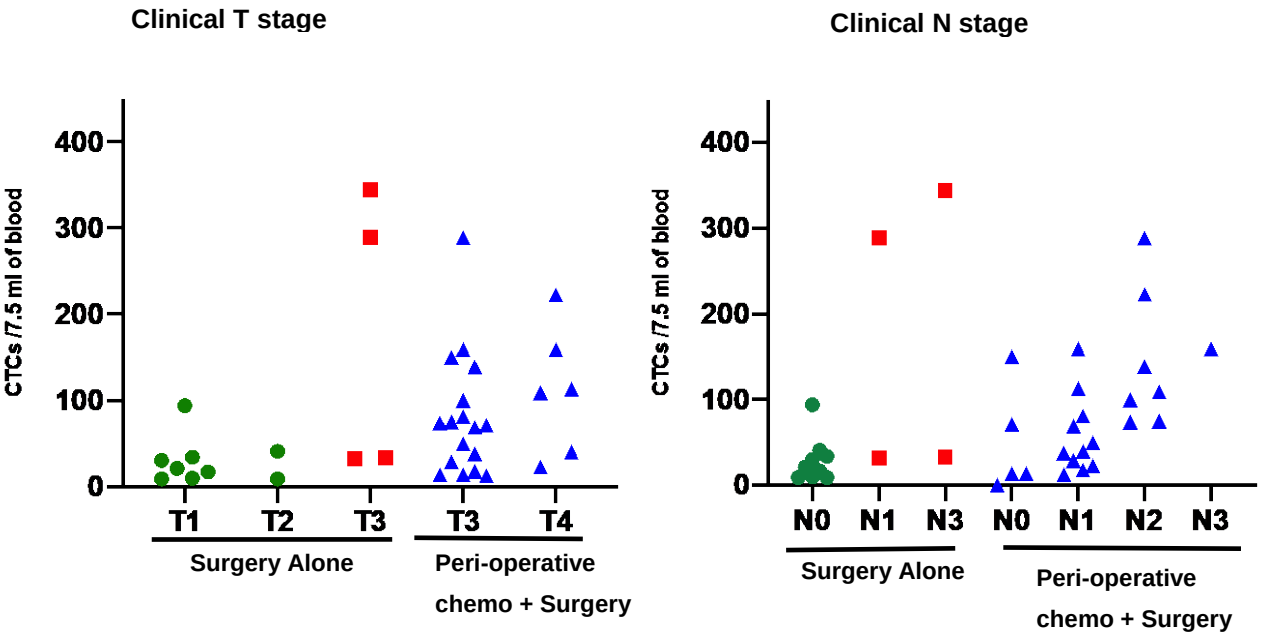
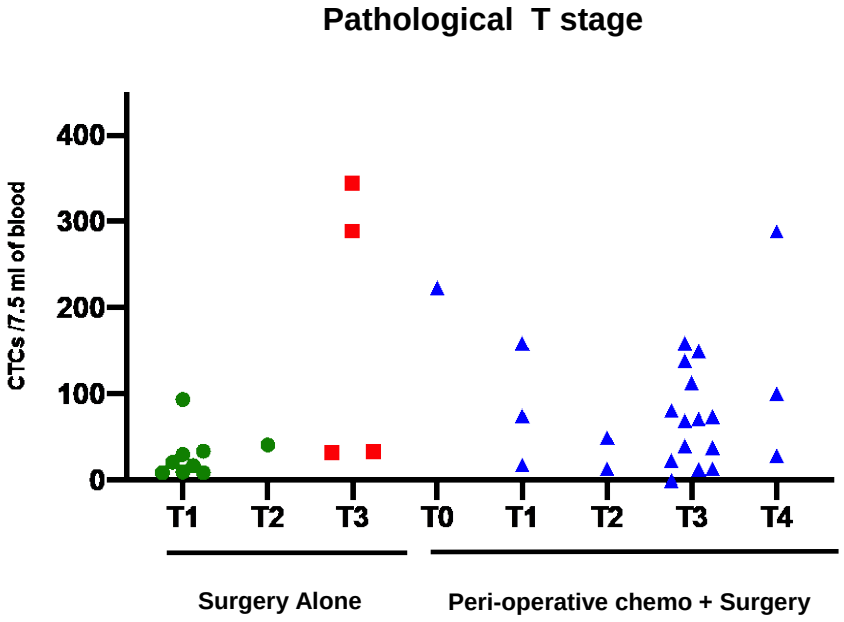


Figure 5.2.7.1– Number of biomarker positive CTCs per 7.5 ml of blood in the pre-operative blood sample and the clinical stage of the disease

All the patient in the early cancer cohort were stage at T2 or < with no evidence of nodal disease. In the locally advanced cancer cohort, there were 7 patients with T4 disease who received peri-operative chemotherapy and surgery of which only one patient had evidence of N3 disease.



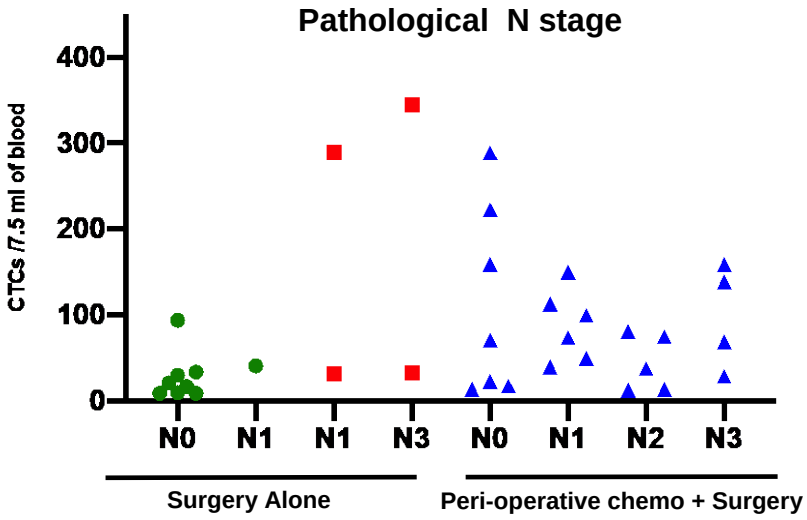


Figure 5.2.7.2– The number of biomarker positive CTCs per 7.5 ml of blood in the pre-operative blood sample and the pathological stage of the disease

Eight out of the nine patients in the early cancer cohort had T1 disease with no evidence of nodal metastases. All the patients in the locally advanced cancer cohort who underwent surgery alone had T3 disease and in two patients there was extensive nodal disease. Six out of the twenty-six patients who received peri-operative chemotherapy and surgery had downstaging of the primary tumour and in one patient there was no tumour visible in the specimen.

There was no significant difference in the number of CTCs in the pre-operative blood sample between the clinical or pathological tumour stage disease (p=0.087). However, the number of postoperative CTCs was higher in patients in whom the primary tumour was staged pathologically as T3 or > compared to primary tumour staged ≤ T2 disease (p=0.002). There was no significant difference in the number of CTCs detected in the pre-operative blood sample in patients with and without nodal metastases (p=0.655). Figure 5.2.7.3 illustrates the correlation between the number of biomarker positive DTCs in the bone marrow and the pathological stage of the disease.

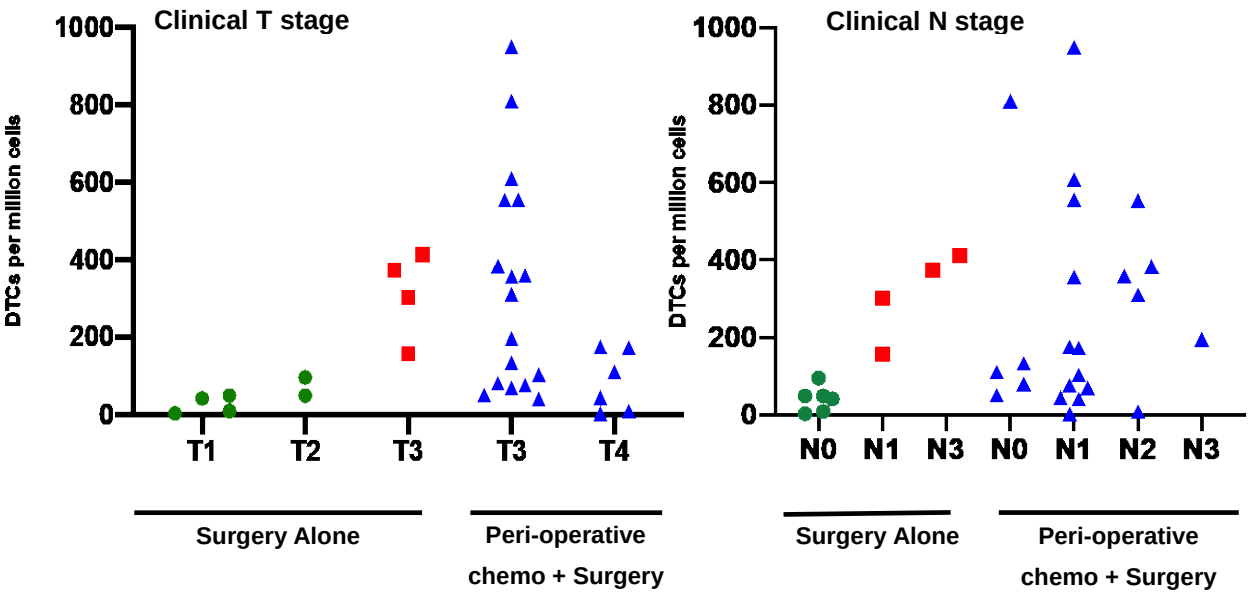


Figure 5.2.7.3– The number of biomarker positive disseminated tumour cells in the bone marrow from the rib and pathological stage of the disease

There were six patients in the early cohort, four patients in the locally advanced cohort who underwent surgery alone and twenty-three in the locally advanced cohort who received peri-operative chemotherapy and surgery who underwent an open oesophagectomy.

Patients with T3 disease or > had higher number of DTCs in the bone marrow when compared to patients early stage cancer ≤ T2 disease in the cohort (p=0.032). Three patients with locally advanced cancer who received peri-operative chemotherapy and surgery had similar number of DTCs in the bone marrow when compared to patients with early clinical stage cancer. Patients with nodal disease had a higher number of DTCs than patients who did not have any evidence of nodal metastases (p=0.041). The presence of lymphovascular invasion (LVI) in the primary tumour is associated with poor prognosis. Figure 5.2.7.4 illustrates the correlation between the number of biomarker positive CTCs in the pre-operative and postoperative blood and the LVI status of the resected specimen.

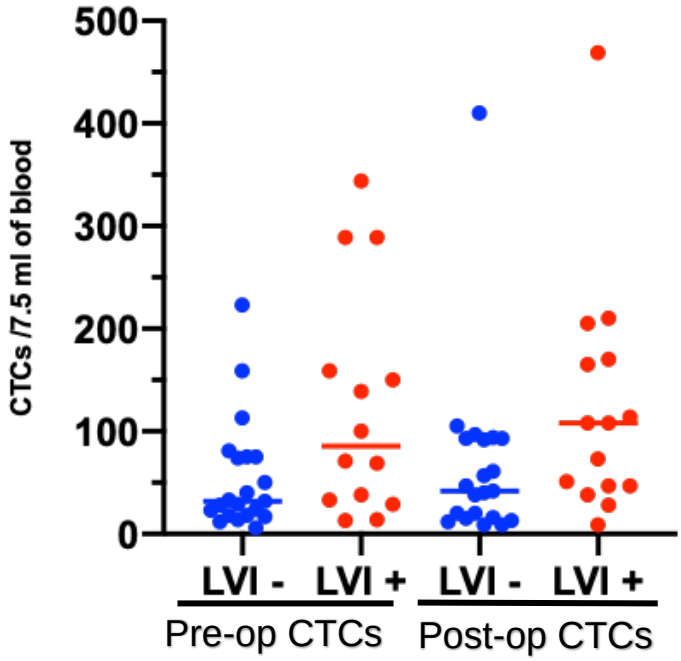


Figure 5.2.7.4– The number of biomarker positive CTCs in the pre and postoperative blood samples in patients based on the LVI status of the primary tumour

There was no evidence of lymphovascular invasion in the primary tumour in patients with early oesophageal cancer. Fifteen patients with locally advanced cancer had evidence of lymphovascular in the primary tumour. The statistical differences between the two groups was tested with Kruskall Wallis test.

There was no significant difference between the number of biomarker positive CTCs in the pre-operative sample in patients in whom the primary tumour had evidence of lymphovascular invasion versus patients in whom the primary tumour did not (p=0.115). The number of biomarker positive CTCs in the postoperative sample was significantly higher than in patients in whom the primary tumour had evidence of lymphovascular invasion (p=0.007). Presence of lymphovascular invasion is an indication of haematogenous and spread of the primary tumour. Such a spread could lead to metastases which could explain the presence of micro metastases in this cohort of patient and thus explain the higher number of CTCs in the postoperative sample despite resection of the primary tumour. Figure 5.2.7.5 compares the number of number of biomarker positive DTCs in patients based on the LVI status of the primary tumour.

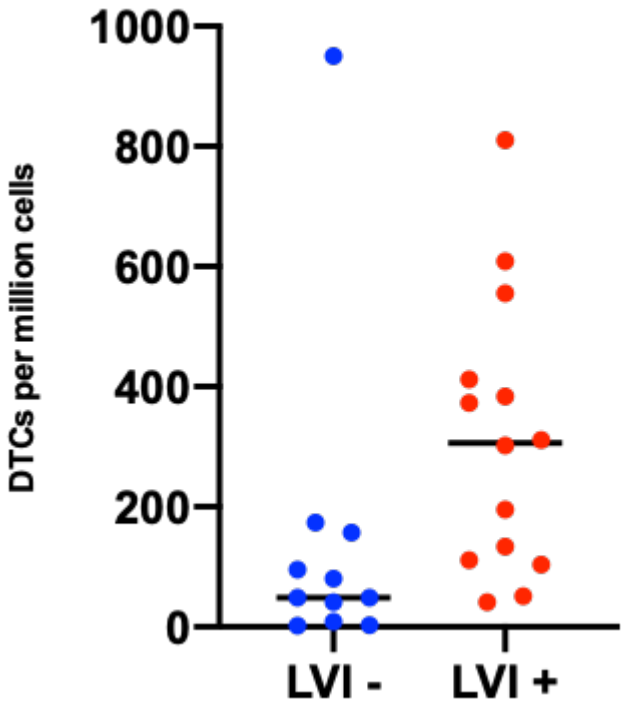


Figure 5.2.7.5 – The number of biomarker positive DTCs in the bone marrow in rib with the lymphovascular invasion status of the primary tumour

There was no evidence of lymphovascular invasion in the primary tumour in patients with early oesophageal cancer. Fifteen patients with locally advanced cancer had evidence of lymphovascular invasion in the primary tumour. The statistical differences between the two groups was tested with Kruskall Wallis test.

The number of biomarker positive DTCs in the bone marrow in rib was higher in patients in whom the primary tumour had evidence of lymphovascular invasion (p=0.001). Positive association between the postoperative CTCs and positive LVI status of the primary tumour in this group patients reflects the greater number of DTCs being present in the bone marrow which acts as reservoir for the tumour cells.

Figure 5.2.7.6 illustrates the correlations between the number of biomarker positive CTCs in the pre-and postoperative blood sample and the tumour regression grade in patients with locally advanced cancer who received peri-operative chemotherapy and surgery.

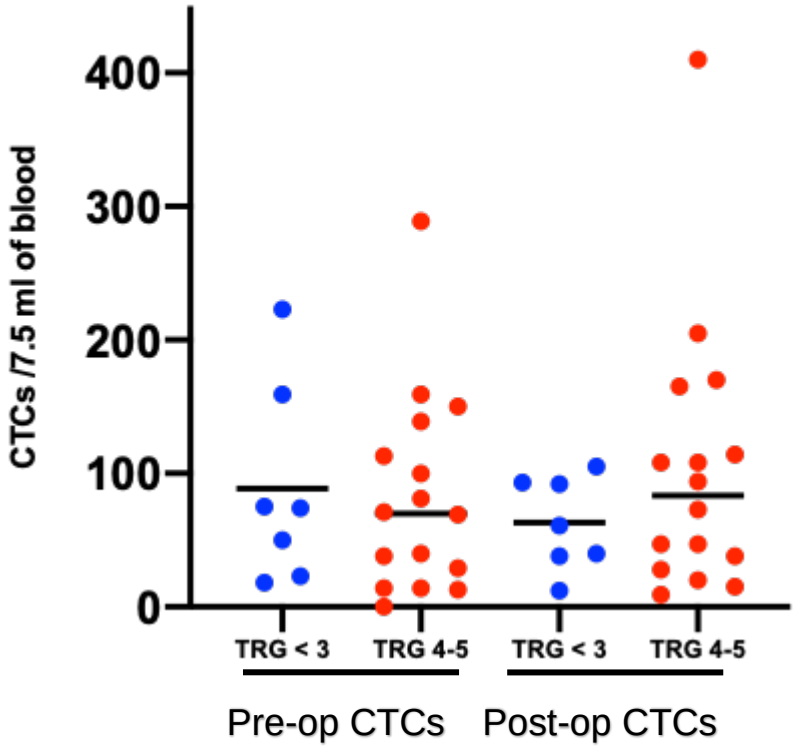


Figure 5.2.7.6– A: Graph comparing the number of pre-operative and postoperative biomarker positive CTCs based on tumour regression grade of the primary tumour

In sixteen patients (67%), the primary tumour was graded at TRG 4-5 which indicated a poor response to peri-operative chemotherapy. The statistical differences between the two groups was tested with Kruskall Wallis test.

There was no significant difference between the number of CTCs in the pre-operative sample in patients who had a good response to perioperative chemotherapy compared to patients with a TRG 4-5 (p=0.344). This applied to the postoperative sample as well (p=0.223). Figure 5.2.7.8 illustrates the correlation between the tumour regression grade and the number of DTCs present in the bone marrow.

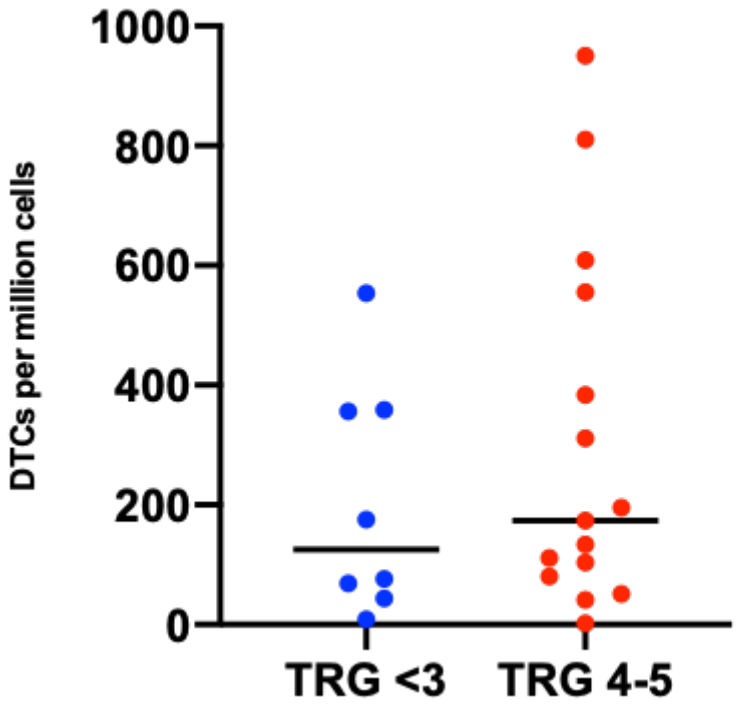


Figure 5.2.7.7– The number of biomarker positive DTCs in the bone marrow and the tumour regression grade of the primary tumour

Eight out of twenty-three patients who received perioperative chemotherapy and an open oesophagectomy had good response to the peri-operative chemotherapy. The statistical differences between the two groups was tested with Kruskall Wallis test.

There was no significant difference between the number of DTCs in the bone marrow of patients who had good response to peri-operative chemotherapy and those who did not (p=0.445). The objectives of peri-operative chemotherapy are to reduce both the local and distant burden of the disease. In combination with surgery, there is an improvement in disease free survival. Poor response to chemotherapy at the distant metastatic sites is one reason that could be responsible for the early relapse in this cohort of patients.

5.2.8 Prognostic significance of circulating and disseminated tumour cells in patients undergoing curative treatment for oesophagogastric cancer

Patients recruited into the study were followed up as part of the protocol for the Northern oesophagogastric unit. Patients were reviewed in clinic at three month intervals in the first year following surgery, six monthly intervals in the second year and annually for the next three years. There is no current evidence for the incorporation of routine imaging in patients with oesophagogastric cancer during the follow up period. In addition, there are no established tumour markers which can be monitored during the follow up period. Patients undergo imaging investigations during the follow up period if they develop clinical symptoms which may indicate recurrence of the cancer, eg progressive weight loss, dysphagia.

Of the 39 patients recruited into the study, there was one in hospital mortality and another patient had metastatic disease at the time of surgery. Both of whom were in the cohort who received peri-operative chemotherapy and surgery. Follow up of the patients in the study is ongoing. The following data on disease free survival is calculated during the period between December 2016 to October 2019. The last patient was recruited into the study in March 2018. The minimum follow up period is 19 months.

Only one patient in the early cancer cohort developed recurrence. However, patient A20 had evidence of nodal disease at the time of surgery. This patient had locally advanced cancer and clinical staging did not identify the presence of nodal disease. The final pathological stage would be Stage III.

Three out of the four patients with locally advanced cancer who underwent surgery alone developed recurrence during the follow up period.

Only 52% (12) of the patients with Stage III and IV cancer received adjuvant chemotherapy following peri-operative chemotherapy and surgery. Fourteen of the twenty-three patients who received peri-operative chemotherapy and surgery developed recurrence in the follow up period. Seven of these patients completed adjuvant chemotherapy.

Figure 5.2.8.1 illustrates the Kaplan Meir curves of two year disease free survival of the three cohorts of patients according to clinical staging.

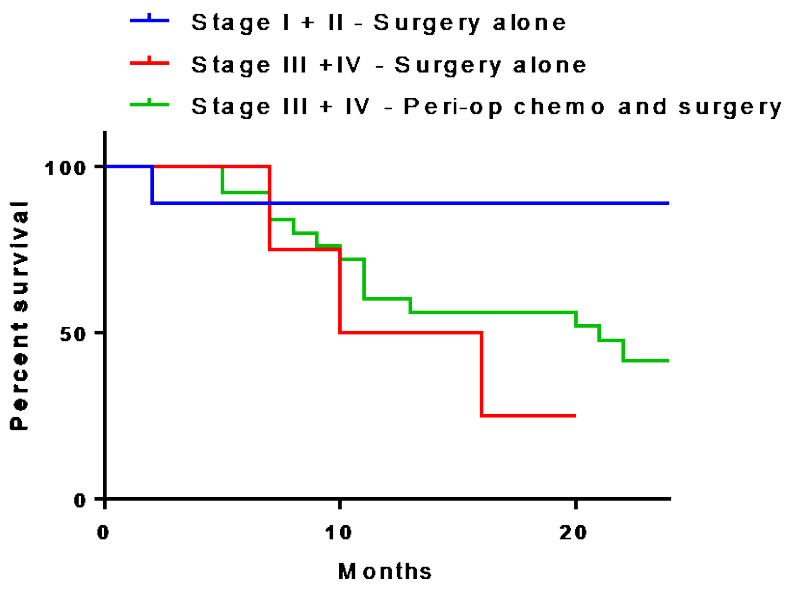


Figure 5.2.8.1. Kaplan Meir curves of two year disease free survival based on the clinical stage of the disease between the three cohort of patients

The median disease free survival in the Stage III and IV patients who underwent surgery alone was 21 months and 23.5 months in Stage III and IV patients who received peri-operative chemotherapy and surgery.

There was no significant difference in disease free survival in patients with stage III and IV disease who received peri-operative chemotherapy and surgery versus surgery alone (p=0.234). Disease free survival at two years in the early cohort was 89%. Of note, this included patient A20 who had locally advanced disease and relapsed during the follow up period. Current literature predicts five year disease free survival at 80% in patients with Stage I and II oesophageal cancer.

Current prognostic markers which predict recurrence are the pathological nodal status, tumour regression grade and the presence of lymphovascular invasion (LVI). There was no incidence of lymphovascular invasion in patients with Stage I and II disease. Three out of the four patients with Stage III and IV disease who underwent surgery alone had evidence of lymphovascular invasion in the specimen. Thirteen out of twenty-four patients with Stage III and IV cancer who received peri-operative chemotherapy had evidence of lymphovascular invasion in the specimen. Six of these patients received adjuvant chemotherapy. Figure 5.2.8.2

illustrates Kaplan Meir analysis of two year disease free survival based on the presence of lymphovascular invasion in the primary tumour.

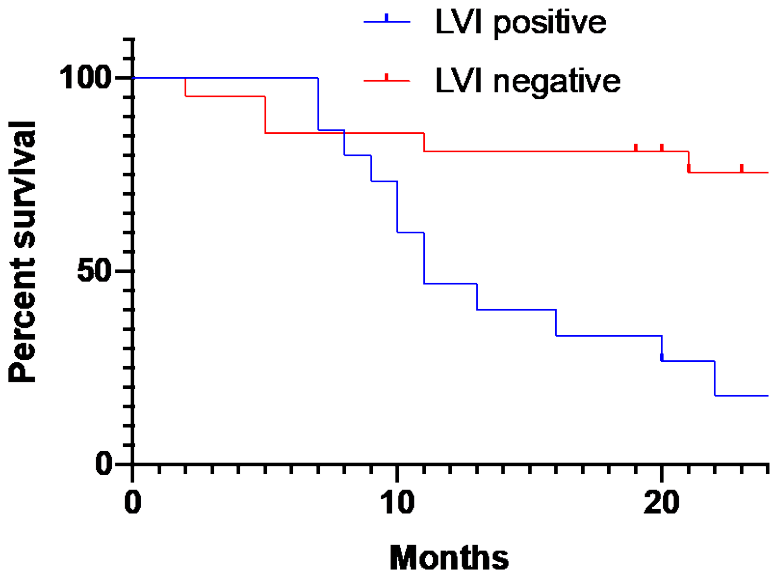


Figure 5.2.8.2. Kaplan Meir curve of two year disease free survival based on the presence of lymphovascular invasion in the pathological specimen

The median survival in patients with the presence of lymphovascular invasion in the pathological specimen was 11 months.

The presence of lymphovascular invasion was a significant prognostic factor in disease free survival (p=0.008).

Only one patient in the early cancer cohort had evidence of nodal involvement following resection. Eight patients with Stage III and IV disease who received peri-operative chemotherapy and surgery had no evidence of macroscopic nodal involvement following surgery. In this cohort, there was one patients who had complete pathological response with no macroscopic evidence of tumour in the resected specimen. The remaining fifteen patients who received perioperative chemotherapy and surgery: six had N1 disease, five had N2 disease and four had N3 disease. All the patients with Stage III and IV disease who underwent surgery alone had evidence of nodal disease: two patients had N1 and two patients had N3 disease. Figure 5.2.8.3 illustrates Kaplan Meir analysis of two year disease free survival based on the presence of nodal metastases.

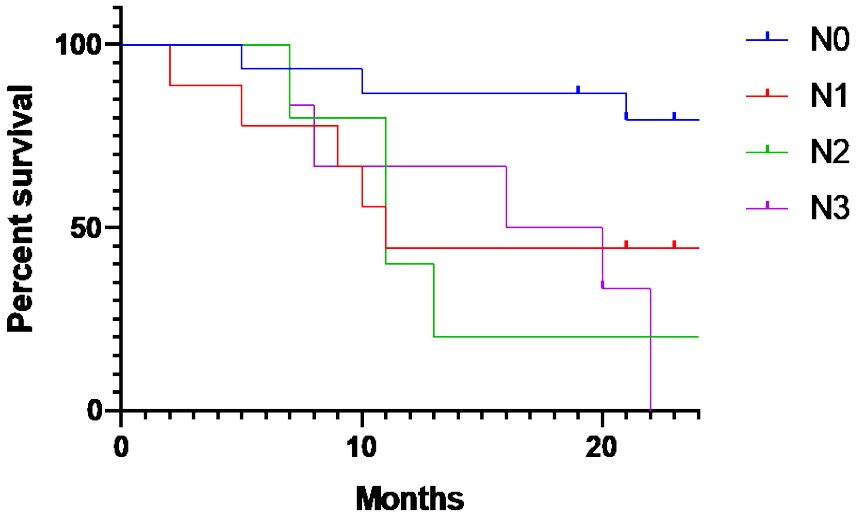


Figure 5.2.8.3. Kaplan Meir curve of two year disease free survival based on the pathological nodal status following resection

The median disease free survival in patient with N1 and N2 nodal disease was 11 months. All patients with N3 disease relapsed within two years following resection.

There was a significant difference in disease free survival between patients with node negative when compared to patients with node positive disease (p=0.023).

Figure 5.2.8.4 illustrate the Kaplan Meir analysis of two year disease free survival based on the tumour regression grade in patients who received peri-operative chemotherapy and surgery.

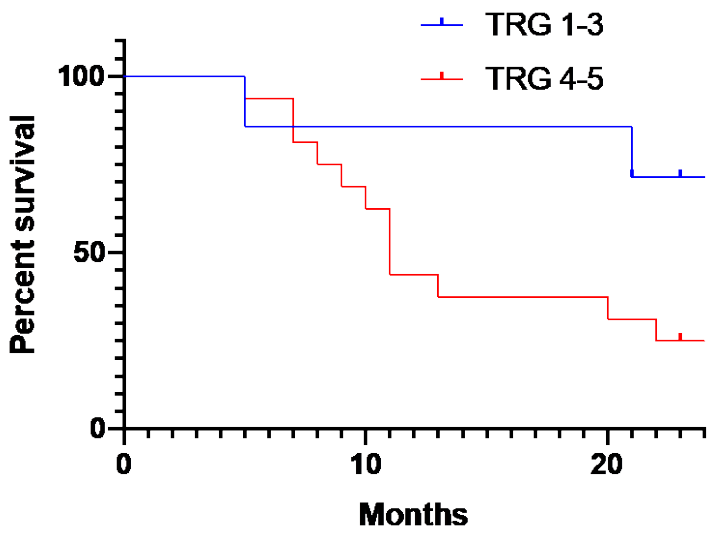


Figure 5.2.8.4. Kaplan Meir curve of two year disease free survival based on the Mandard tumour regression grade

Sixteen of the twenty-three patients in this cohort had evidence of poor response to chemotherapy, tumour regression grade 4 or higher. The median disease free survival in this cohort was 11 months. There was significant difference in survival based on the TRG (p=0.009).

The two year disease free survival was higher in patients with tumour regression grade 3 or less which indicates a good response to peri-operative chemotherapy.

Patients were stratified into cohorts based on the number of CTCs present in blood in the pre and postoperative sample. The number of CTCs in each cohort was not normally distributed. The median number of CTCs per 7.5 ml of blood which expressed biomarkers in the pre-operative blood sample was 30 (2-93) and 31 (4-137) in the postoperative sample in patients with early oesophageal cancer. In the locally advanced cohort, the median number of CTCs per 7.5 ml of blood which expressed biomarkers in the pre-operative blood sample was 84 (5-284) and 86 (4-428) in the postoperative sample in patients with locally advanced cancer. Based on this data, the patients were divided into four cohorts based on the number of CTCs per 7.5 ml of blood which expressed a biomarker present in the pre and postoperative sample: less than or equal to 49 CTCs, 50-99 CTCs, 100 to 149 CTCs and greater than 150 CTCs. Figure 5.2.8.5 and Figure 5.2.8.6 illustrates the Kaplan Meir survival analysis based on these four cohort of patients.

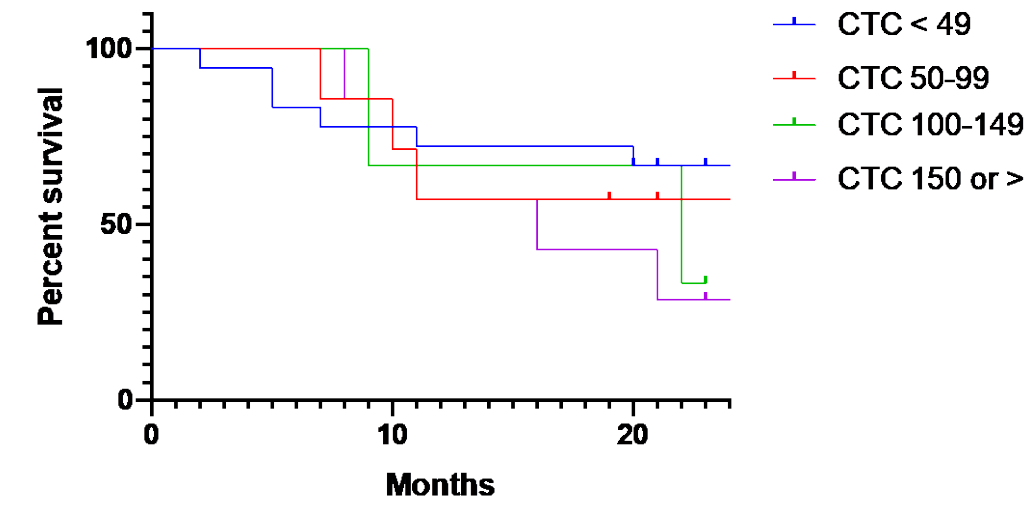


Figure 5.2.8.5. Kaplan Meir analysis of two year disease free survival based on the number of CTCs per 7.5 ml of blood present in the pre-operative blood sample in patients who underwent curative treatment for oesophageal cancer

The median survival in patients with less than 49 CTCs per 7.5 ml of blood and 50 – 100 CTCs per 7.5 ml of blood was not reached, 100-149 CTCs per 7.5 ml of blood was 22 months and 150 or more CTCs per 7.5 ml of blood was 16 months. There was no significant difference between the four cohorts (p=0.711)

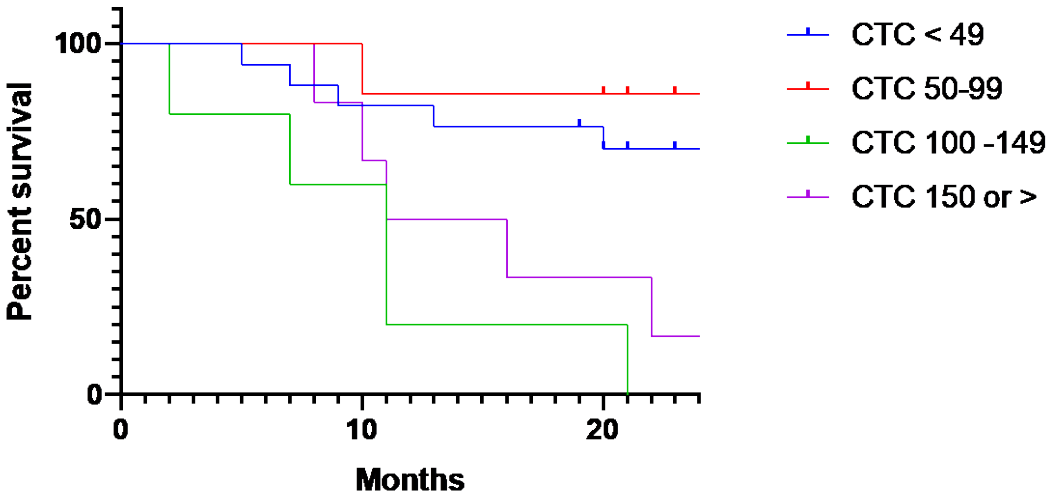


Figure 5.2.8.6 Kaplan Meir analysis of two year disease free survival based on the number of CTCs per 7.5 ml of blood present in the post-operative blood sample in patients who underwent curative treatment for oesophageal cancer

The median survival in patients with less than 49 CTCs per 7.5 ml of blood was not reached, 50-99 CTCs per 7.5 ml of blood was 31 months, 100-149 CTCs per 7.5 ml of blood was 11 months and 150 or more CTCs per 7.5 ml of blood was 13.5 months. There was a significant difference between the four cohorts (p=0.001)

Eighteen patients had fewer than 49 CTCs per 7.5 ml of blood in the pre-operative sample. Of these eighteen patients, eight had early cancer and two had locally advanced cancer and underwent surgery alone. The remaining eight patients had received peri-operative chemotherapy and surgery. In the pre-operative sample, there was no difference in survival between patient who had less than 100 CTCs per 7.5 ml of blood and patients who had more than 100 CTCs per 7.5 ml of blood (p=0.408). In the postoperative sample, eight out of the eighteen patients with less than 50 CTCs per 7.5 ml of blood had early oesophageal cancer. The remaining ten patients received peri-operative chemotherapy and surgery for locally advanced cancer.

All the patients with Stage I and II cancer based on the pathological specimen had less than 50 CTCs per 7.5 ml of blood in the blood. There was a significant difference in survival in patients who had more than 100 CTCs and those who had less than 100 CTCs per 7.5 ml of blood in the post-operative sample (p=0.002). Kaplan Meir survival analysis was performed based on the expression of epithelial biomarkers in CTCs in the pre and postoperative sample.

There was no difference in survival based on expression of individual epithelial biomarkers in CTCs in the pre-operative sample (p=0.488). However, in 50% of patients there were two different populations of CTCs in the pre-operative sample which expressed epithelial biomarkers (EpCAM and/or cytokeratins). Twenty-five patients had only one population of CTCs which expressed an epithelial biomarkers, of which ten patients had CTCs which expressed both EpCAM and cytokeratins. Figure 5.2.8.7 illustrates Kaplan Meir analysis of two year disease free survival comparing the two cohort of patients described.

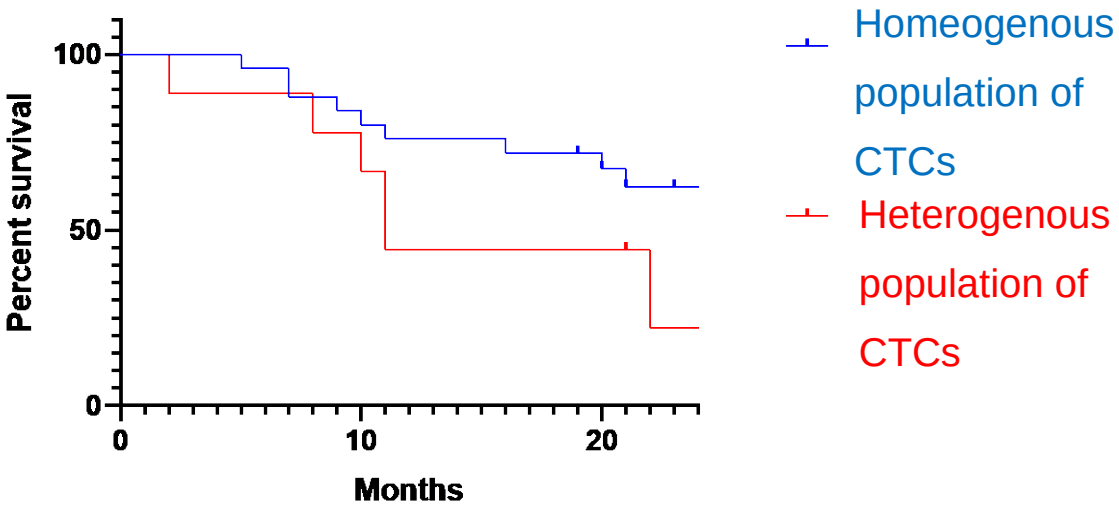


Figure 5.2.8.7. Kaplan Meir survival analysis of two year disease free survival based on the number of populations of CTCs which express either individual or multiple epithelial biomarkers in the pre-operative blood sample

The presence of two or more populations of CTCs which expressed epithelial biomarkers was associated with poor survival, median time to relapse 16 months in this cohort.

The presence of two or more populations of CTCs which expressed epithelial biomarkers in the pre-operative sample was associated with a significant decrease in disease free survival (p=0.043).

In the postoperative sample, 48% of the patients had only one population of CTCs which expressed epithelial biomarkers of which 59% of patients had CTCs which expressed EpCAM or cytokeratins.

Figure 5.2.8.8 illustrate Kaplan Meir analysis of two year disease free survival based on the number of populations of CTCs present in each postoperative sample.

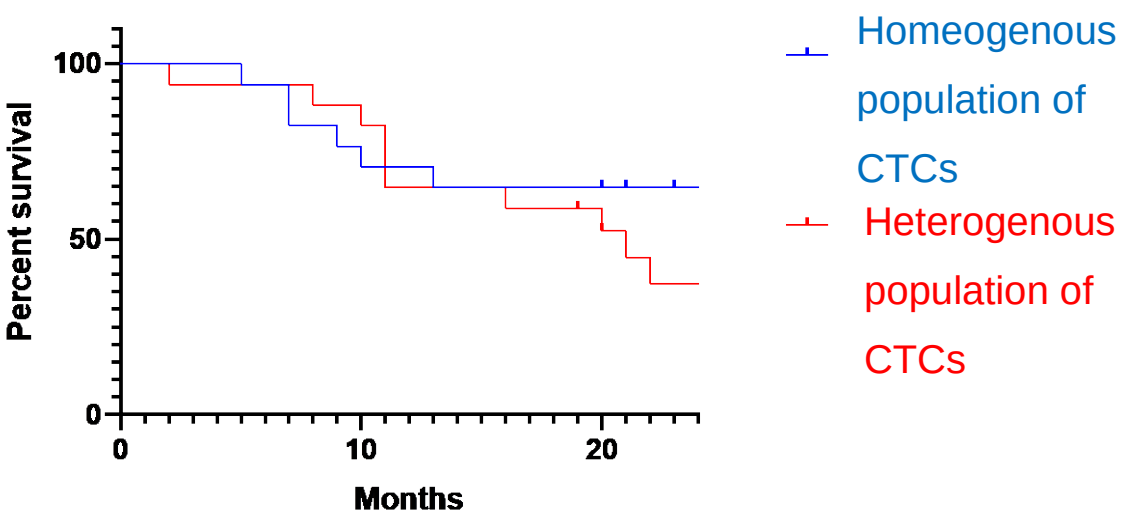


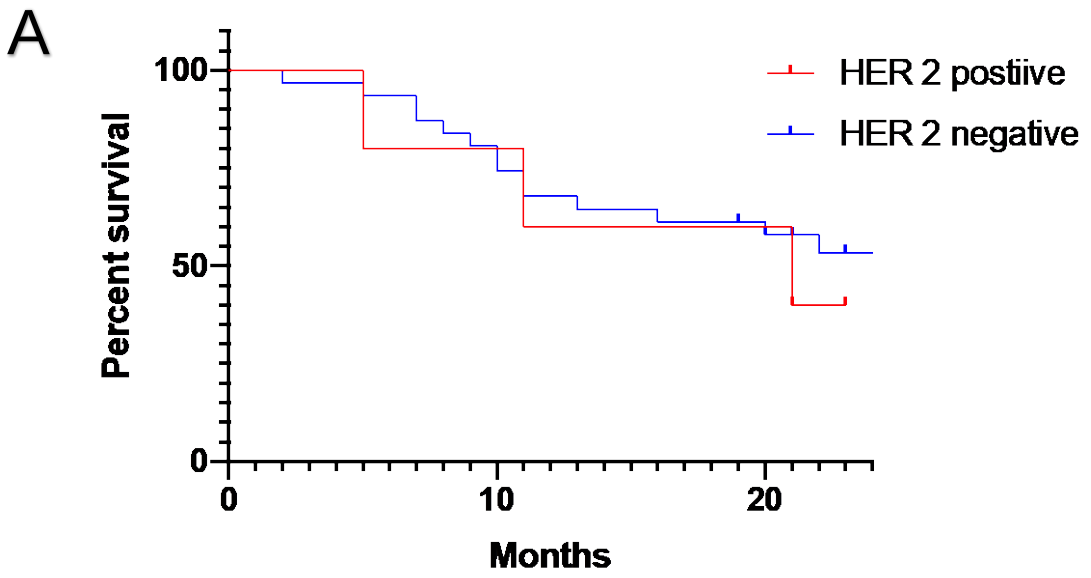
Figure 5.2.8.8. Kaplan Meir analysis of two year disease free survival based on the number of populations of CTCs which express either individual or multiple epithelial biomarkers in the postoperative blood sample

There were 17 patients who had two or more population of CTCs which expressed two or more different epithelial biomarkers. The median survival in group of patients with two or more populations of CTCs was 21 months.

In the postoperative sample, the number of patients with two or more populations of CTCs which expressed epithelial biomarkers was the same as in the pre-operative sample. However, there was no significant difference in disease free survival between the two cohort of patients based on the expression of epithelial biomarkers by CTCs in the postoperative sample (p=0.198).

The most common novel biomarkers detected in CTCs in the pre and postoperative samples were HER2 and c-Met. There were six patients in the pre-operative sample who had CTCs which expressed HER2, four of whom received peri-operative chemotherapy and surgery. Patient A14 had two populations of CTCs both of which expressed HER2, one population expressed HER2 and cytokeratins and the other expressed EpCAM and HER2. There were seven patients with CTCs that expressed HER2 in the postoperative sample. Two of these patients had CTCs that expressed HER2 in the pre-operative sample. Two patient, A6 and A40 had CTCs which expressed HER2 in the postoperative sample but no pre-operative blood sample was available to make a direct comparison. Patient A33 had CTCs that expressed HER2

in the postoperative sample but was excluded from the final survival analysis due to in hospital mortality. Figure 5.2.8.9 illustrates the survival based on the CTC expression of HER2 in the pre and postoperative blood sample.



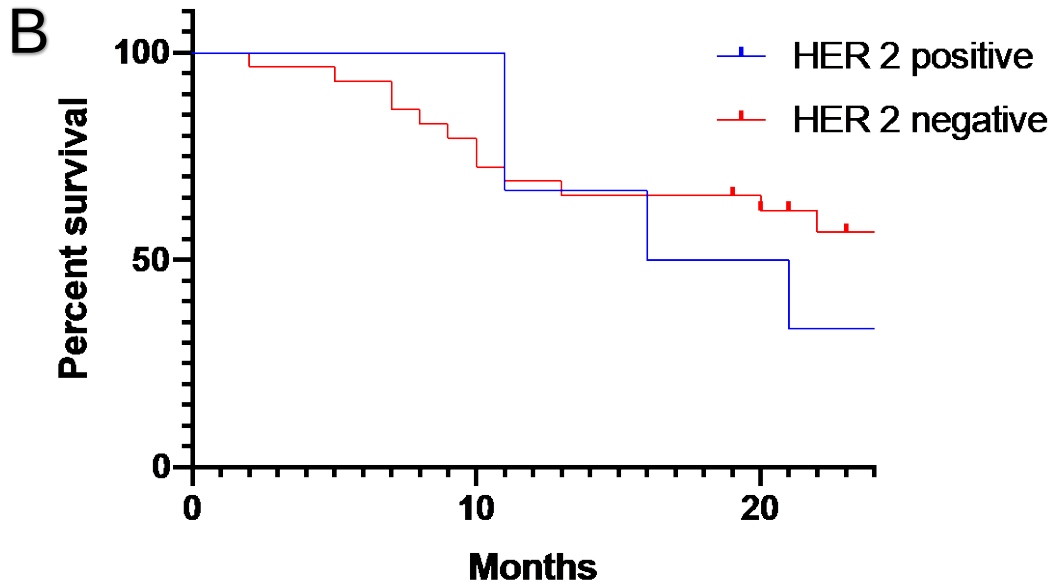


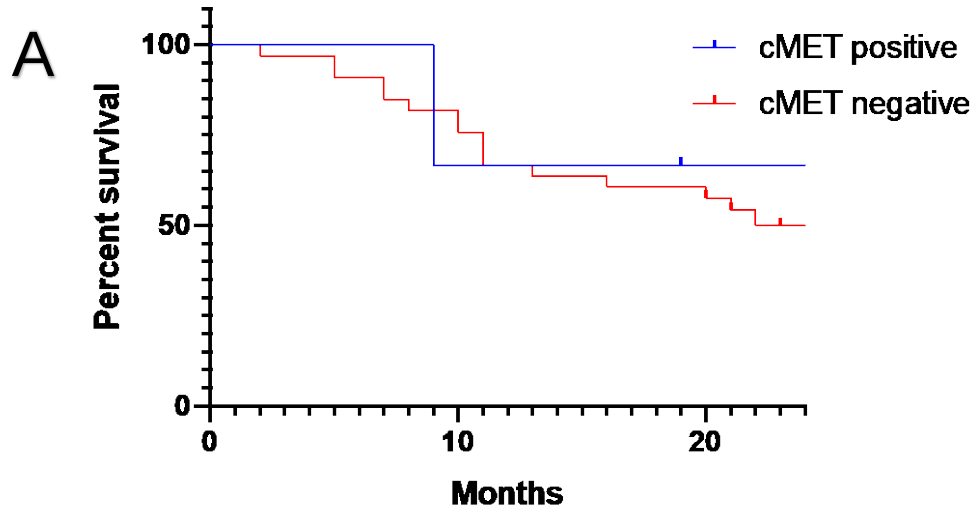
Figure 5.2.8.9. Kaplan Meir analysis of two year disease free survival based on the expression of HER2 in CTCs.

A: Pre-operative blood sample B: Postoperative blood sample

Three out of the four patients with CTCs which expressed HER2 in the pre-operative sample relapsed in 20 months or less following surgery. The median time to relapse was 11 months. In the postoperative sample, five out of the seven patients relapsed during the follow up period. The median time to relapse was 18.5 months.

The two year disease free survival was not significantly different in patients with CTCs which expressed HER2 in either the pre (p=0.208) or postoperative samples (p=0.314).

Three patients had CTCs which expressed cMET in the pre-operative sample, two of whom received peri-operative chemotherapy and surgery. Only two patients had CTCs which expressed cMET in the postoperative sample. Figure 5.2.8.10 illustrates the Kapalan Meir survival analysis based on the CTC expression of c-Met in the pre and postoperative blood sample.



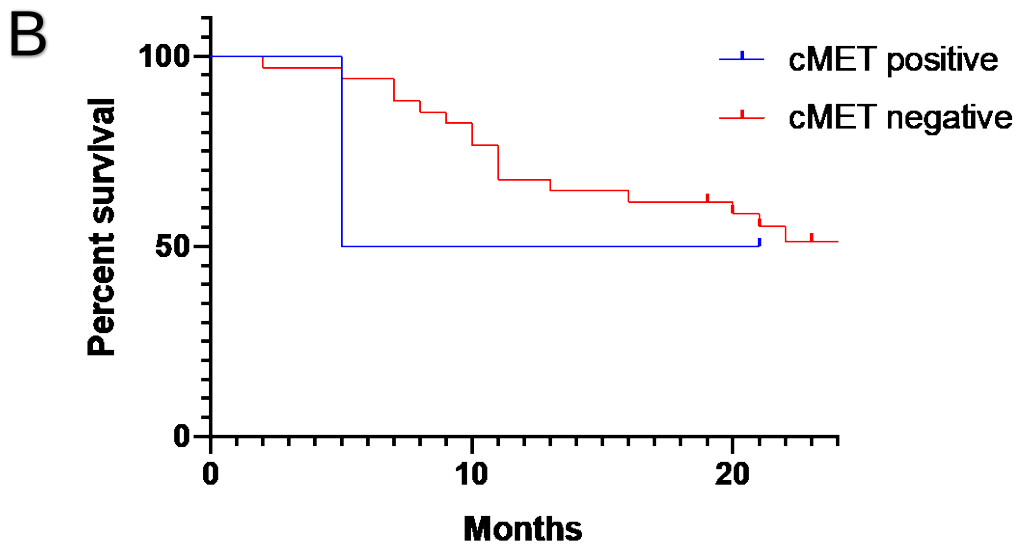


Figure 5.2.8.10. Kaplan Meir analysis of two year disease free survival based on the expression of c-Met in CTCs.

A: Pre-operative blood sample B: Postoperative blood sample

In both the pre and postoperative samples, there was no difference in disease free survival between patients who had CTCs that expressed c-Met versus patients with CTCs which did not.

Thirty-two (82%) patients underwent an open oesophagectomy of whom six patients had early oesophageal cancer and twenty-two patients received peri-operative chemotherapy and surgery for locally advanced cancer. As stated in the previous section, DTCs were detected in all patients undergoing curative treatment for oesophageal cancer. The number of DTCs detected in each cohort varied and there was heterogeneity in biomarker expression within the DTC populations. Figure 5.2.8.11 illustrate the Kaplan Meir analysis of two year disease free survival based on the different populations of DTCs detected in each bone marrow sample.

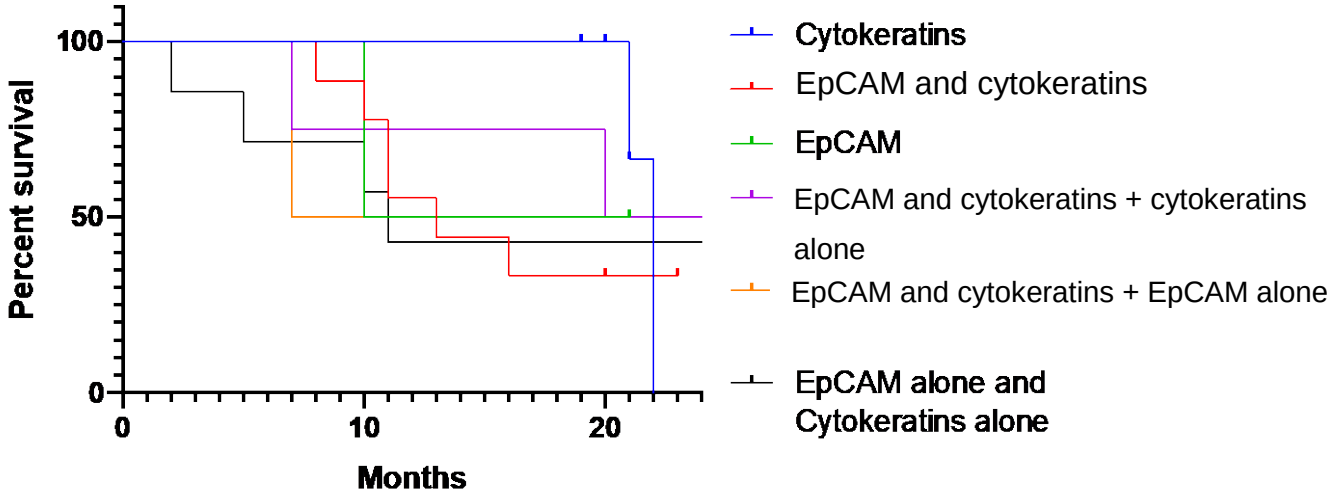


Figure 5.2.8.11. Kaplan Meir analysis of two year disease free survival based on the expression of epithelial biomarkers by DTCs in the bone marrow

Nine patients (28%) had DTCs which expressed both EpCAM and cyokeratins (CellSearch™ criteria) alone. Seven patients (22%) of patients had two populations of DTCs which expressed epithelial markers, one population expressed cytokeratins alone and the other EpCAM alone.

There was no difference in survival based on the expression of either individual or multiple biomarkers in DTCs in the bone marrow (p=0.767).

Twenty-two patients had populations of DTCs which expressed one or more of the novel biomarkers. Figure 5.2.8.12 illustrate the Kaplan Meir survival analysis based on the expression of the novel biomarkers in the DTCs detected in the bone marrow.

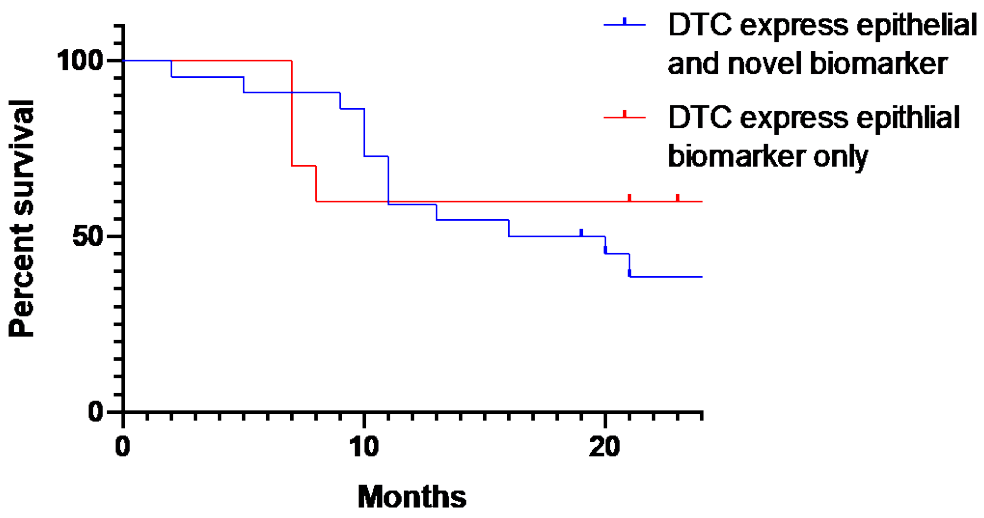


Figure 5.2.8.12. Kaplan Meir analysis of two year disease free survival based on DTCs which express epithelial and novel biomarkers versus DTCs which express epithelial biomarkers alone

Twenty-two (69%) of patients had DTCs which expressed both epithelial and novel biomarkers. The median survival in this cohort of patients was 18 months versus 31 months in patients who had DTCs which expressed epithelial biomarkers.

There was no significant difference in survival between patients who had DTCs which expressed an epithelial and a novel biomarker compared to patients with DTCs which expressed epithelial biomarkers only (p=0.968). Figure 5.2.8.13 illustrate Kaplan Meir survival analysis based on the expression of the novel biomarkers expressed in the DTCs.

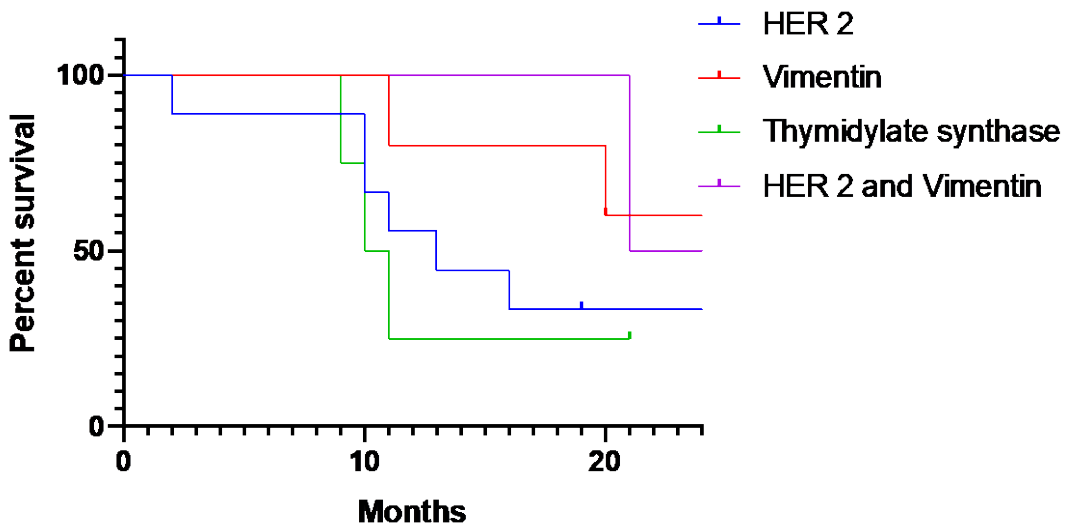


Figure 5.2.8.13. Two year disease free survival based on DTCs which express the novel biomarkers: HER2, Vimentin, Thymidylate Synthase

Nine patients (41%) had DTCs that expressed HER2 only and two patients had DTCs that expressed HER2 and vimentin. Five patients (23%) had DTCs that expressed Vimentin and four patients (18%) had DTCs that expressed thymidylate synthase. The median survival in patients with DTCs which expressed HER2 was 13 months and thymidylate synthase was 10.5 months.

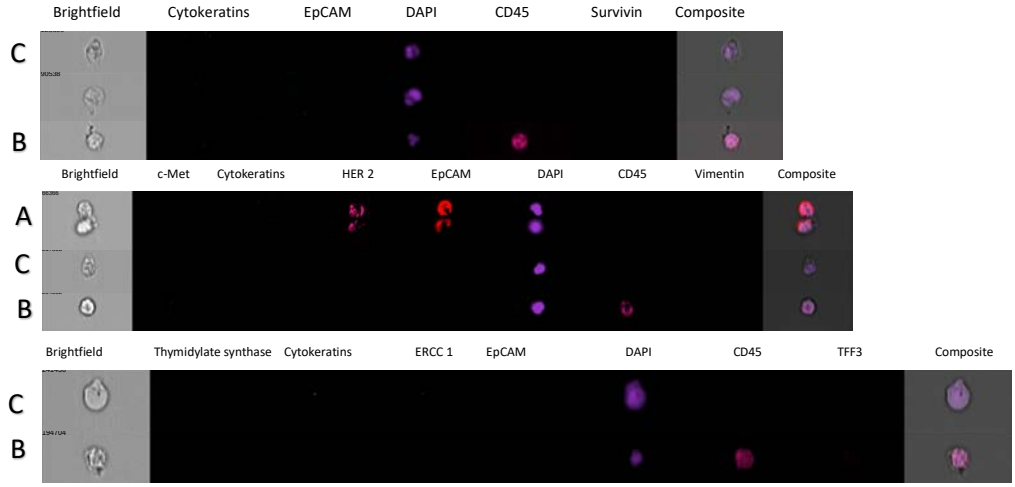
No significant difference in survival was present between the different populations of DTCs that expressed novel biomarkers.

5.2.9 Circulating tumour cells in patients with early recurrence following curative treatment

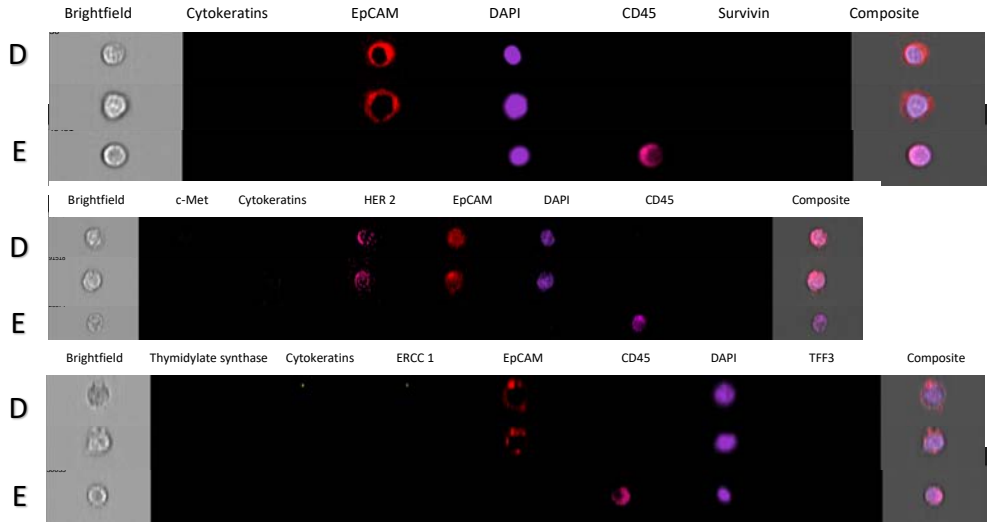
Fourteen out of the twenty-three patients who received peri-operative chemotherapy and surgery relapsed in the follow up period. The median time to recurrence in this cohort was 20 months. Patient A10 received peri-operative chemotherapy and surgery for a junctional oesophageal adenocarcinoma. Postoperative histology indicated a good response to chemotherapy and no evidence of nodal metastases. The patient developed local recurrence confirmed radiologically within five months of surgery and presented with malignant pleural effusion. Figure 5.2.9.1 illustrates images of the CTCs identified in the pre and postoperative sample, CTCs identified in the blood at the time of recurrence and tumour cells in the pleural effusion.

A10– T4N1 – Peri-operative chemotherapy and surgery

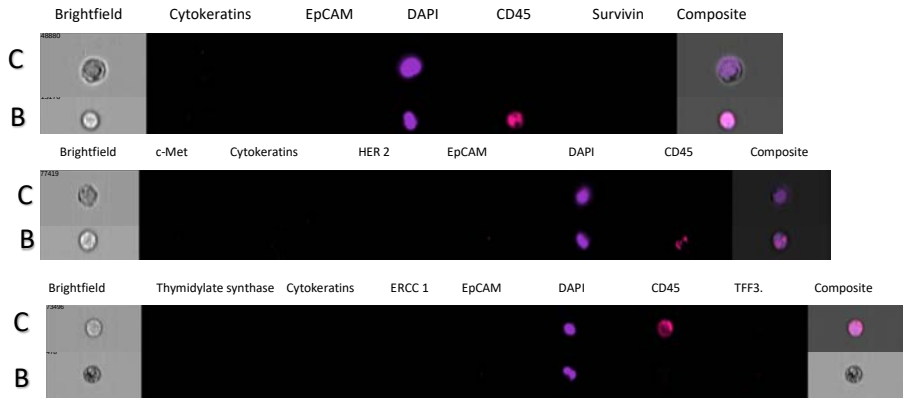
CTCs in pre-operative sample



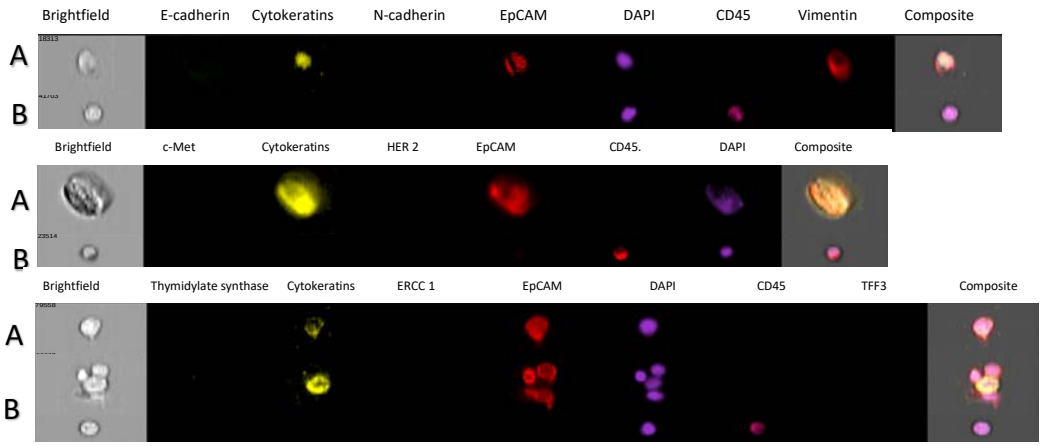
DTCs in the bone marrow of excised rib



CTCs in the postoperative sample



CTCs in blood sample following relapse



Tumour cells in the pleural effusion

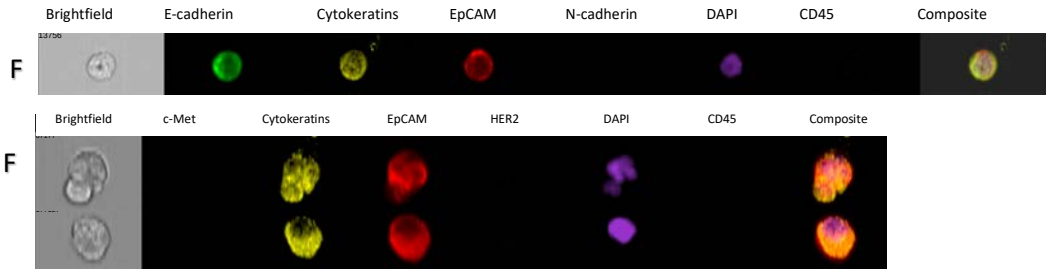


Figure 5.2.9.1. Images of CTCs in the blood – pre-operative, postoperative sample and at the time of recurrence, DTCs in the bone marrow of excised rib and tumour cells in the pleural effusion detected in patient A 10

A: Biomarker positive CTCs detected in the blood from pre-operative sample, postoperative sample and taken at time of relapse B: white blood cells in the blood from pre-operative sample, postoperative sample and taken at time of relapse C: Biomarker negative CTCs detected in the blood from pre-operative sample, postoperative sample and taken at time of relapse D: Biomarker positive DTCs in the bone marrow of excised rib E: white blood cells in the bone marrow of excised rib F: tumour cells in the pleural effusion post following relapse

In the pre-operative blood sample, 58 CTCs per 7.5 ml of blood were identified of which 17 expressed EpCAM and HER2 and the remainder did not express any of the biomarkers in the panels.

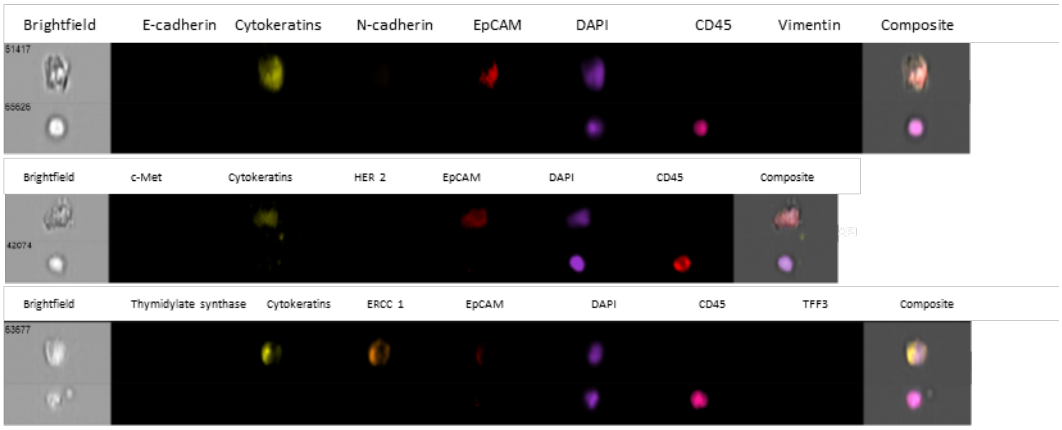
In the postoperative sample, there were 40 biomarker negative CTCs per 7.5 ml of blood identified. There were 52 CTCs per 7.5 ml of blood that were biomarker positive in the blood sample taken following recurrence: 16 CTCs expressed EpCAM, cytokeratins and vimentin and the remainder all expressed EpCAM and cytokeratins. There were two populations of tumour cells identified in the pleural effusion, one populations expressed EpCAM and cytokeratin and the other expressed E cadherin, EpCAM and cytokeratins.

Patient A10 had low numbers of CTCs in both the pre and postoperative samples. In addition, there was no evidence of poor prognostic factors based on the pathology. Early relapse at the site of surgery in combination with the presence of a malignant pleural effusion indicates transcoelomic spread of the tumour. Of interest, one population of CTCs in the blood and the tumour cells in pleural effusion share similar biomarker expression. This evidence suggests that one population of CTCs may orginate from the pleural metatastes.

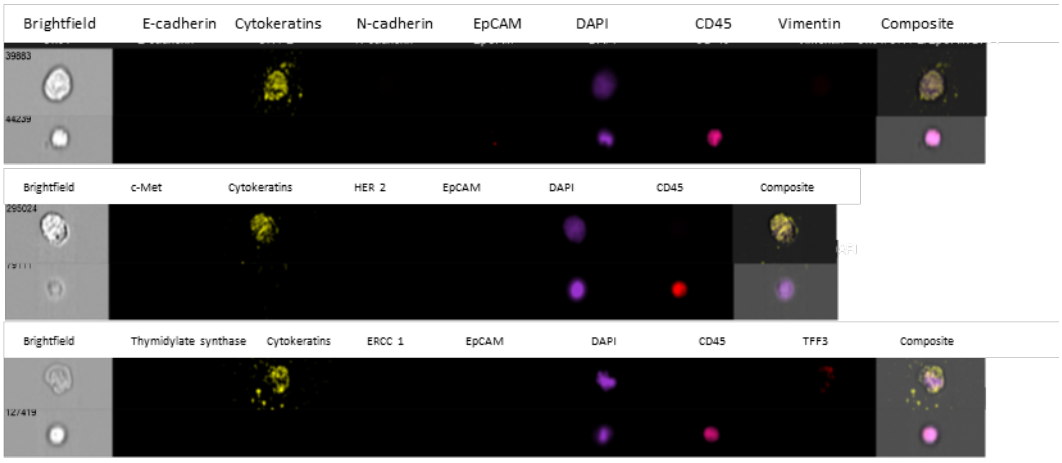
Patient A20 underwent an open oesophagectomy for early oesophageal cancer, clinical stage was T2N0. However, there was evidence of nodal spread at the time of surgery and the pathological stage was T2N1. The patient relapsed within two months of surgery with evidence of pleural effusion and local recurrence. Figure 5.2.9.2 illustrates images of the CTCs identified in the pre and postoperative blood samples and CTCs identified in the blood at the time of recurrence.

A20 – T2N0 – Surgery Alone

CTCs in pre-operative sample



CTCs in post operative sample



CTCs in the blood sample post recurrence

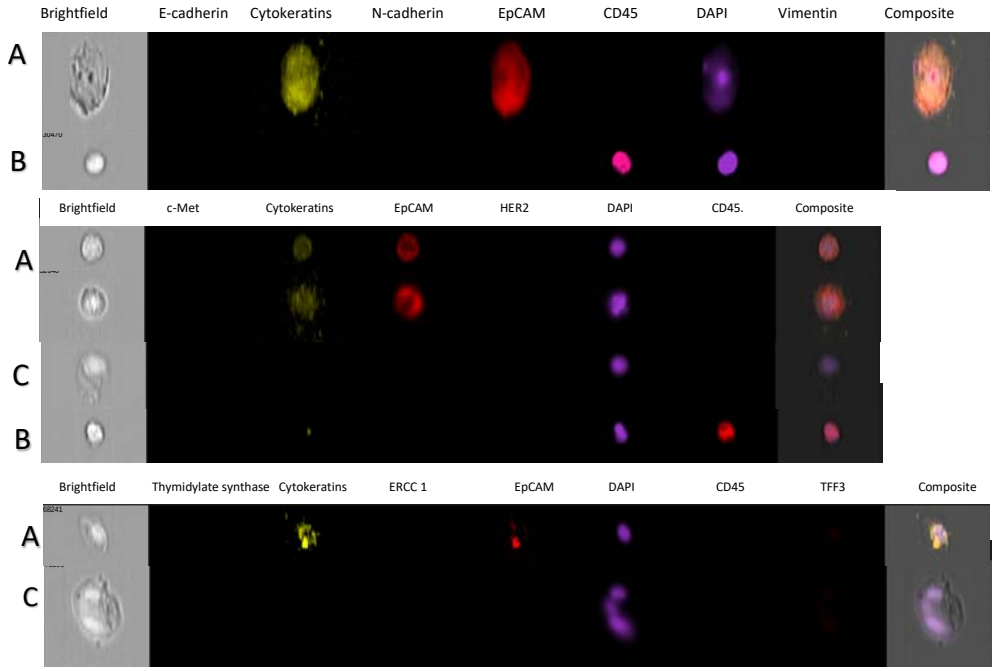


Figure 5.2.9.2. Images of CTCs in the blood in the pre-operative sample, postoperative sample and in blood after radiological evidence of relapse in patient A20

A: Biomarker positive CTCs detected in the blood from pre-operative sample, postoperative sample and taken at time of relapse B: white blood cells in the blood from pre-operative sample, postoperative sample and taken at time of relapse C: Biomarker negative CTCs detected in the blood from pre-operative sample, postoperative sample and taken at time of relapse

In the pre-operative blood sample, 49 CTCs per 7.5 ml of blood were identified of which 45 expressed EpCAM and cytokeratins. There two populations of DTCs in the bone marrow, one expressed cytokeratin alone and the other expressed EpCAM and cytokeratins. In the postoperative sample, there were 171 CTCs per 7.5 ml of blood which expressed cytokeratin only. Blood sample taken at the time following recurrence, identified approximately 76 CTCs per 7.5 ml of blood which expressed EpCAM and cytokeratins. Pleural sample was not obtained for this patient and thus correlation with the tumour cells in the effusion is not possible. The number of CTCs in the blood sample at the time of recurrence was lower than in the immediate postoperative period. However, there was a different population of CTCs present in the blood at this point when compared to the immediate postoperative period. The presence of DTCs which expressed both EpCAM and cytokeratin could indicate the origin of the CTCs from this site.

5.3 Discussion

Two studies detected CTCs in only 20% of patients undergoing treatment for oesophageal adenocarcinoma (128, 129). In these studies, cells in the blood were classified as CTCs if they expressed both EpCAM and cytokeratins. The high resolution imaging flow cytometer enables the detection of cells in the blood based on the morphology of the cells and the expression of either individual or multiple biomarkers. Cells in the study were classified as a CTC if they expressed one or more epithelial and/or mesenchymal and/or novel biomarkers and/or morphologically similar to CTCs based on surface area and hyperpolidy. CTCs were detected in all patients undergoing curative treatment for oesophageal adenocarcinoma independent of the stage of the tumour based on this classification criteria. There were five populations of CTCs identified based on biomarker expression. In the pre-operative sample, positive expression of both EpCAM and cytokeratins were present in 50% of CTCs in patients with early cancer who underwent surgery alone, 50% of CTCs in patients with locally advanced cancer who underwent surgery alone and 25% of CTCs in patients with locally advanced cancer who received peri-operative chemotherapy and surgery alone. In the post operative sample, positive expression of both EpCAM and cytokeratins were present in 28% of CTCs in patients with early cancer who underwent surgery alone, 40% of CTCs in patients with locally advanced cancer who underwent surgery alone and 30% of CTCs in patients with locally advanced cancer who received peri-operative chemotherapy and surgery alone expressed both EpCAM and cytokeratins. The second most abundant population of CTCs expressed EpCAM only, the third population of CTCs expressed cytokeratins only and the fourth population expressed the novel biomarkers (HER2, thymidylate synthase, c-Met, Vimentin) and/or an epithelial biomarker. The fifth population of CTCs identified consisted of cells which did not expressed any biomarker but were morphologically similar to CTCs. In patients who received peri-operative chemotherapy and surgery, the cells accounted for 36% of the total CTC population in the pre-operative sample and were present in 10 patients. In the postoperative sample, 12 patients had biomarker negative CTCs in the blood and they accounted for 48% of CTCs detected in this group.

Patients with advanced disease based on both clinical and pathological staging had a higher number of CTCs in the blood and DTCs in the bone marrow. LVI is a prognostic factor in

survival. The number of CTCs in the postoperative blood sample and DTCs in the bone marrow were higher in patients with evidence of lymphovascular invasion in the lymph nodes (p=0.223)(p=0.051). Patients with poor response to peri-operative chemotherapy based on the TRG 4-5 have a higher number of CTCs in the postoperative sample (p=0.344). Both these observations indicate the presence of micrometastases in these patients and one can postulate that the postoperative CTCs are arising from a site of distant metastases.

In previous studies, the presence of one or more CTCs was reported to be of prognostic significance in patients undergoing curative treatment for oesophageal adenocarcinoma. In these studies, cells are classified as CTCs if they expressed both EpCAM and cytokeratins. In this study, CTCs are classified based on their morphology, size, nuclear density and individual biomarker expression. As a consequence, the number of CTCs detected is higher than documented previously. Furthermore, there was no significant difference in the number of CTCs in the pre-operative sample between patients with early and locally advanced cancer according to clinical stage. Differentiating between these two groups of patients based on individual number of CTCs in the preoperative sample alone is not possible. The rise or fall in number of CTCs between the pre and postoperative sample provides important prognostic information. The number of CTCs in the postoperative sample was higher in patients in who the primary tumour had evidence of lymphovascular invasion (p=0.007).

Approximately 61% of patients with locally advanced cancer who received peri-operative chemotherapy and surgery relapsed within two years. Prognostic information was determined from the results based on the presence of CTCs in the blood and DTCs in the bone marrow combined with the presence of heterogeneity within these populations. Patients with two or more different population of CTCs based on epithelial biomarker expression was associated with a significant reduction in two year disease free survival in the pre-operative blood sample (p=0.043). The presence of heterogeneity within the CTC population reflects the complex interaction between the primary tumour and the surrounding environment. Improved understanding of the morphology of the CTCs will aid in identifying the origin of the cells and their role in tumour development. In addition, detection of novel biomarkers such as HER2 and c-Met could identify potential therapeutic targets for peri- operative and adjuvant treatment for oesophageal adenocarcinoma.

Minimally invasive surgery in oesophageal cancer has been shown to be associated with lower number of CTCs in the intra and postoperative period. In this study, three patients underwent minimally invasive surgery. There was no difference in the number of CTCs detected between the minimally invasive and open cohort of patients in this study.

The prognostic significance of the change in the number of CTCs in the postoperative period remains unknown. Previous studies have documented a rise in the CTCs in the immediate postoperative period being associated with poor prognosis in patients undergoing curative treatment for lung, gastric and colorectal cancer (156-158). However, studies with a longer follow up period have shown that the CTC levels fall and return to levels similar or less than in the pre-operative period (159). In the present study, patients with over 100 CTCs per 7.5 ml of blood in the postoperative blood sample had a significantly lower two year disease free survival ($p=0.001$) compared to patients with less than 100 CTCs per 7.5 ml of blood.

Chapter 6: Circulating and disseminated tumour cells in patients undergoing palliative treatment for oesophagogastric cancer

6.1 Introduction

The majority of patients with oesophagogastric cancer present with disease that is not amenable to curative treatment. There are three main reasons why curative treatment is not considered. Firstly, the disease is locally advanced with the tumour invading adjacent structures and/or there is the presence of nodal involvement outside the surgical resection field. Secondly, there are distant metastases present commonly in the lung, liver or bone. A proportion of patients who are diagnosed with distant metastases present with symptoms secondary to the presence of metastatic disease, eg ascites secondary to peritoneal disease. Thirdly, patients who are unfit for radical curative treatment due to their medical co-morbidities. The main aims of palliative treatment are to alleviate symptoms related to the cancer, reduce the systemic burden of the disease and improve survival. Palliative treatment modalities are focused on achieving these three main aims. Palliative chemotherapy aims to treat local and systemic disease. Chemotherapy regimens can vary dependant on the histological subtype of the tumour and its location. Patients with metastatic junctional oesophagogastric and gastric adenocarcinoma undergo HER2 testing of the primary tumour consequent to the results of the TOGA trial (51). The TOGA trial showed a survival benefit with trastuzumab and chemotherapy versus chemotherapy alone, in patients with metastatic junctional oesophageal adenocarcinoma with HER2 overexpression of the primary tumour, median survival 13.8 months versus 11.1 months. Palliative radiotherapy aims to treat the primary cancer within the oesophagus or oesophagogastric junction. The aim is to reduce the size of the tumour and improve symptoms of dysphagia. Radiotherapy is used in the emergency setting to treat bleeding arising from the primary tumour. Other palliative treatment modalities aim to manage the symptoms that arise as a result of metastatic disease. The ascites secondary to peritoneal disease which is treated with paracentesis. A stent will alleviate the symptoms of dysphagia caused by an obstructing oesophageal cancer. A proportion of patients with medical co-morbidities present with metastatic oesophagogastric cancer. Poor functional status and the presence of co-morbidities preclude this group of

patients from receiving palliative chemotherapy and/or radiotherapy. Symptom control with medication is the mainstay of treatment.

6.2 Results

6.2.1 Circulating tumour cells in patients with advanced oesophagogastric cancer undergoing palliative treatment

Patients were recruited into the study under two different ethics with the associated consent forms and patient information sheets. Ethics approval for the study was obtained with the Health Research Authority NHS England (appendix A) and the Newcastle university biobank (appendix C). Patients were identified at the Northern Oesophagogastric cancer MDT and at the oesophagogastric ward 36, Royal Victoria infirmary, Newcastle upon Tyne. The inclusion criteria for the study were patients over the age of 18 with either locally advanced and/or metastatic oesophagogastric adenocarcinoma undergoing palliative treatment. The study was discussed with the patient and an information sheet was provided. Patients were entered into the study following completion of the consent form (Appendix B) with the principal investigator. Blood samples were collected prior to the start of palliative treatment.

Exudates in the form of pleural and ascitic fluid were collected in sterile containers and transferred to the laboratory in a safety bag. Pathological data were obtained from the primary tumour. Details of the palliative treatment regimen was obtained from the Northern Centre for Cancer Care (NCCC).

The study incorporated a wide range of patients with metastatic oesophagogastric cancer. Patients with the identifier commencing with P were recruited under the Ethics form, IRAS ID 18535 (appendix A). The remainder of the patients were recruited under the Newcastle biobank ethics form. Patient P6 was excluded from the data analysis due to degradation of the blood sample following collection.

Table 6.2.1.1 illustrates the demographics of the patients recruited into the study, location of the primary tumour, site of metastases and the palliative treatment regimen.

Table 6.2.1.1 Demographics of patients recruited into the study undergoing palliative treatment for primary metastatic oesophagogastric cancer.

Identifier Number	Age	Sex	Tumour Site	Site of Metastases	Tumour stage – T	Nodal Stage - N	HER2 status	Exudate	Palliative treatment modality
P2	60	F	Oesophagogastric junctional (GOJ) Type II	Liver Metastases	T4	N2	Positive	No	Chemotherapy
P3	68	M	Oesophagogastric junctional (GOJ) Type III	Pulmonary metastases	T4	N3	Positive	No	Chemotherapy
P4	74	F	Oesophagogastric junctional (GOJ) Type II	Retroperitoneal nodes	T4	N3	Positive	No	Chemotherapy
P5	61	M	Oesophagogastric junctional (GOJ) Type II	Isolated Peritoneal nodule	T4	N3	Negative	No	Chemotherapy
P7	58	M	Oesophagogastric junctional (GOJ) Type II	Liver Metastases	T4	N2	Positive	No	Chemotherapy
P8	60	M	Distal Oesophageal		T4	N3	Negative	No	Radiotherapy
P9	64	M	Proximal Gastric	Peritoneal metastases	T4	N2	Negative	No	Chemotherapy
P10	64	F	Proximal Gastric	Peritoneal metastases	T4	N2	Negative	Ascites	Chemotherapy
P11	70	F	Proximal Gastric	Liver metastases	T4	N2	Negative	No	Chemotherapy
SH1	82	M	Oesophagogastric junctional (GOJ) Type II	Peritoneal metastases	T4	N3	Positive	Ascites	Best supportive care

BB1	85	M	Oesophagogastric junctional (GOJ) Type II	Peritoneal metastases	T4	N3	Negative	Ascites	Best supportive care
JT	35	F	Gastric Linitis	Peritoneal metastases	T4	N2	N/A	No	Chemotherapy

Table 6.2.1.2 Demographics of patients recruited into the study undergoing palliative treatment for oesophagogastric cancer following recurrence after previous surgery.

Identifier Number	Age	Sex	Previous Surgery	Site of recurrence	HER 2 Status	Exudate	Palliative Treatment modality
P1	80	M	Sub-total Oesophagectomy 2002 - T2N1 – Adenocarcinoma	Liver Metastases	Negative	No	Chemotherapy
JR	64	M	Sub-total Gastrectomy - 2016 T4N3 Adenocarcinoma	Peritoneal metastases and local recurrence - 2017	Negative	Ascites	Chemotherapy

Seven out of twelve patients in this study had metastatic primary oesopahgogastric junctional adenocarcinoma. In five of these patients, the primary tumour had evidence of HER2 expression. Chemotherapy was the mainstay of the palliative treatment in this group of patients. Patient P8 had a distal oesophageal cancer with anaemia secondary to bleeding at the site of the primary tumour and hence underwent radiotherapy. There were two patients who had developed recurrence post curative treatment. Patient P1 underwent an oesophagectomy in 2002 for a Stage II adenocarcinoma of the oesophagus and developed liver metastases in 2016. Patient JR underwent a sub-total gastrectomy for Stage IV adenocarcinoma of the distal stomach in January 2017. Patient JR had received neo-adjuvant and adjuvant chemotherapy. There was evidence of peritoneal recurrence with ascites in September 2017 following emergency admission with abdominal pain.

Patients SH1 and BB1 presented with ascites secondary to peritoneal disease. Both patients had multiple medical co-morbidities and poor functional status. They were unfit for palliative treatment.

6.2.2 Characterisation of circulating tumour cells in patients with metastatic oesophagogastric cancer

Two patients, SH1 and BB1 had over 800 CTCs per 7.5 ml of blood. These patients had evidence of metastatic adenocarcinoma of the gastrooesophageal junction and were medically unfit for palliative treatment. Figure 6.2.2.1 illustrates CTCs identified in blood sample obtained from patient SH1 who had a HER2 positive junctional oesophagogastric adenocarcinoma Figure 6.2.2.2 illustrates CTCs identified in the blood sample from patient BB1 who had a HER2 negative junctional oesophagogastric adenocarcinoma.

SH1 - T4N3M1 – Junctional oesophagogastric adenocarcinoma with ascites

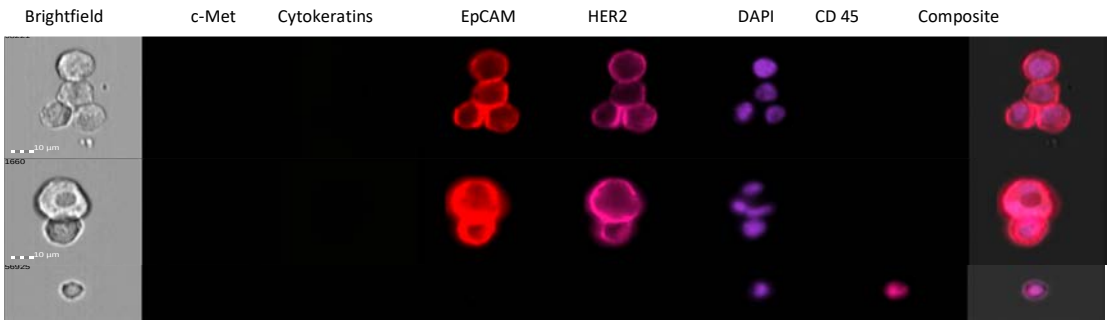


Figure 6.2.2.1 – Images of CTCs and a white blood cells identified by high resolution imaging flow cytometer in the blood sample in patient SH1

A large proportion of the CTCs were present in clusters. The CTCs were significantly larger than the white blood cells. There was only one population of CTCs which expressed EpCAM and HER2. The primary tumour in patient SH1 was HER2 positive.

BB1 - T4N3M1 – Junctional oesophagogastric adenocarcinoma with ascites

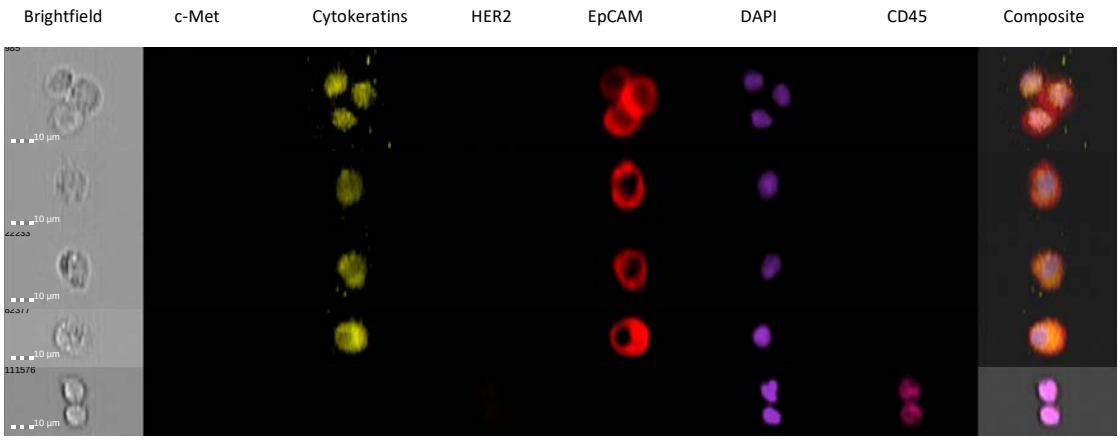


Figure 6.2.2.2– Images of CTCs and a white blood cells identified by high resolution imaging flow cytometer in the blood sample in patient BB1

There were CTC clusters present in the blood sample. The primary tumour was HER2 negative. One population of CTCs identified in the sample which expressed EpCAM and cytokeratins. The CTCs did not express any of the other biomarkers in the panel.

The presence of large number of CTCs combined with the presence of CTC clusters reflects presumably the stage of advanced oesophagogastric malignancy in patients who did not receive any treatment. CTC clusters were identified in five out of 12 patients being treated with palliative intent. All of the patients had evidence of metastatic disease. Of note, in all five patients the blood samples were taken prior to the commencement of any palliative treatment.

Figure 6.2.2.3 illustrates the number of circulating tumour cells identified in patients with metastatic oesophagogastric cancer.

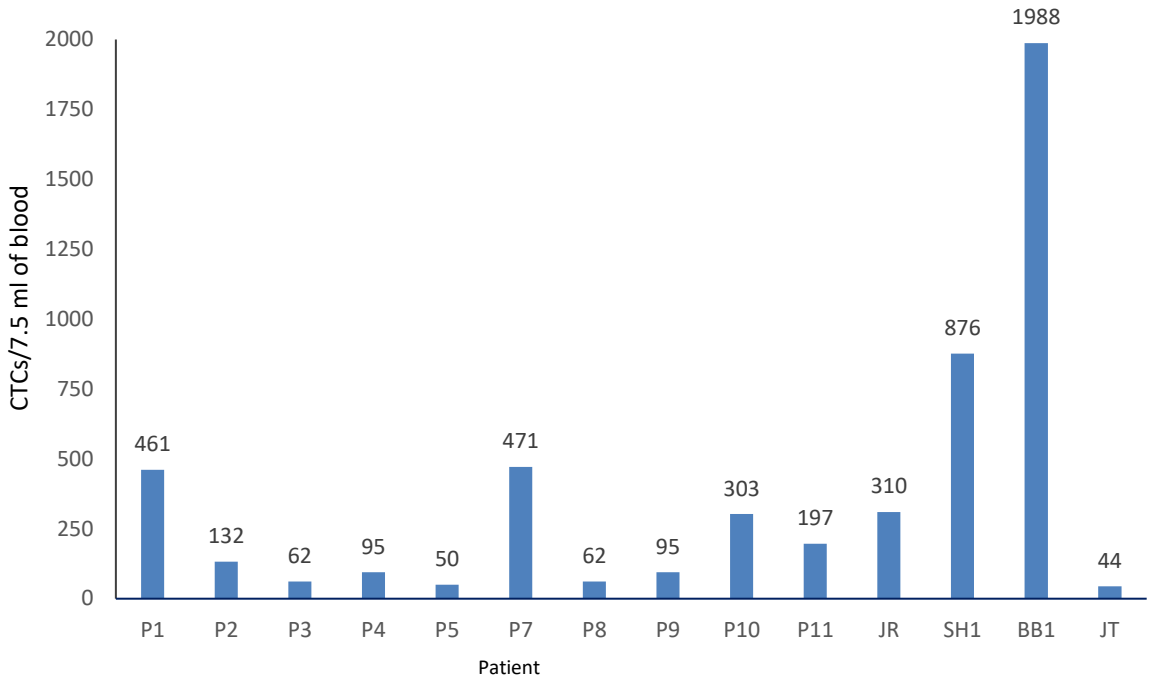


Figure 6.2.2.3 – The number of circulating tumour cells in patient with metastatic oesophagogastric cancer in 7.5 ml of blood

There is a wide variation in the number of CTCs present in this cohort of patients. Of note, patient JT had fewer than 50 CTCs per 7.5 ml of blood in his blood which is comparable to patients undergoing curative treatment as illustrated in Chapter 5. The patients in the palliative cohort can be grouped based on the location and extent of metastatic disease. Patients with locally advanced disease are not amenable to curative resection if there is evidence of macroscopic nodal metastasis outside the field of curative resection or if there is local invasion of the tumour into surrounding structures. Isolated peritoneal nodules and ascites indicate the presence of transcoeloemic spread. Liver metastases indicate the presence of haematogenous spread. Figure 6.2.2.4 illustrates the sub-group analysis of CTCs detected in this group of patients based on the location and extent of the metastatic disease.

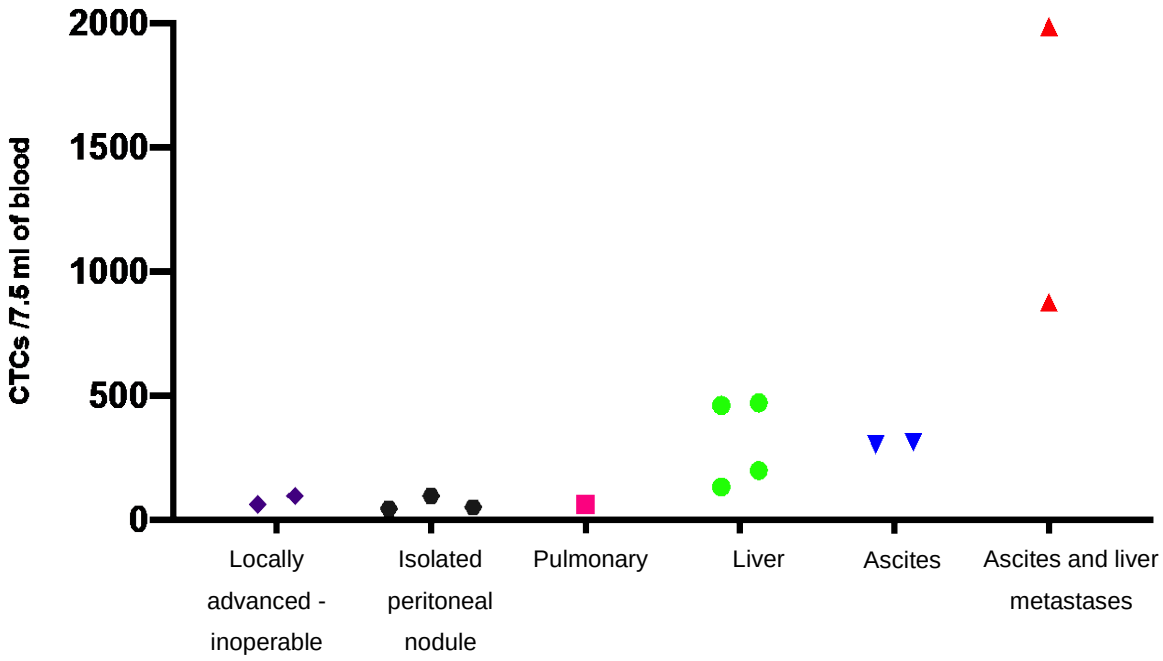


Figure 6.2.2.4 – The number of circulating tumour cells in patients with metastatic oesophagogastric cancer in 7.5 ml of blood based on the location and extent of metastatic disease

The number of CTCs correlates with the location and burden of metastatic disease. Four patients had ascites secondary to the presence of extensive peritoneal metastases. In patients with distant metastases and/or ascites, the number of CTCs were higher than in the patients with locally advanced disease (p=0.021).

Two patients had isolated peritoneal nodules detected on staging laparoscopy with histological confirmation of metastases. Patients with ascites had a significantly higher number of CTCs detected in the blood when compare to patients with isolate peritoneal nodules (p=0.029)

Two patients had evidence of macroscopic nodal disease based on radiological imaging outside the field of resection and were deemed inoperable. There was no histological confirmation of the presence of nodal metastasis in these patients. There was no difference in the number of CTCs in this group of patients with locally advanced disease deemed inoperable compared to patients with locally advanced cancer undergoing curative treatment with surgery alone (p=0.344).

In the majority of the patients in this group, there was evidence of heterogenous population of CTCs present in the blood sample. Figure 6.2.2.5 illustrates the biomarker expression of CTCs in patients with metastatic junctional oesophagogastric cancer.

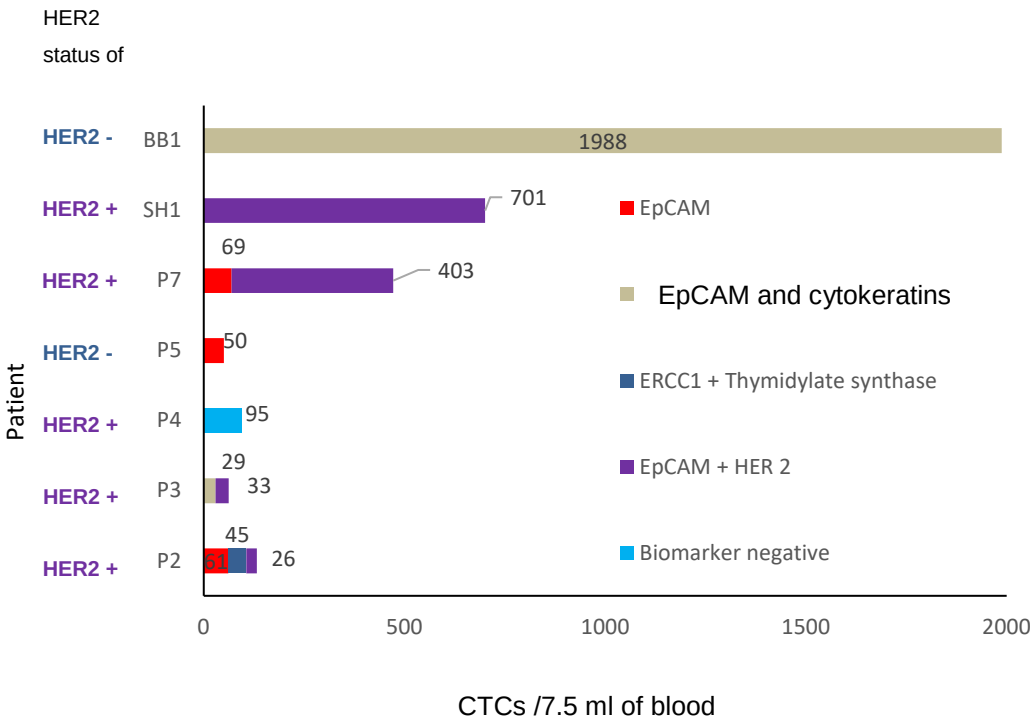


Figure 6.2.2.5 – Biomarker expression of CTCs in patients with metastatic oesophagogastric junctional adenocarcinoma

Patients BB1 and SH1 had the highest number of CTCs in the study and both had only one population of CTCs present. There was strong correlation with the biomarker expression of HER2 in the CTCs in the blood and the primary tumour. CTCs which expressed HER2 were present in 5 out of 7 patients with oesophgagogastric junctional adenocarcinoma whose primary tumour was HER2 positive. The primary tumour in Patient P4 was HER2 positive but the CTCs in the blood did not express any of the biomarkers in the panels tested.

Figure 6.2.2.6 illustrates the biomarker expression of CTCs in patients with metastatic gastric cancer

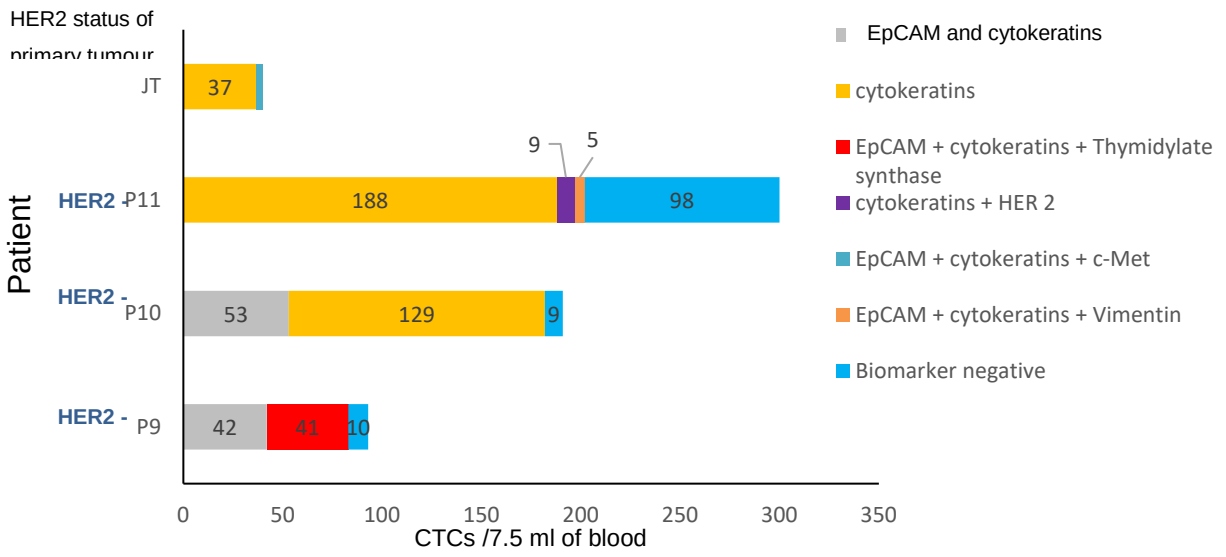


Figure 6.2.2.6 – Biomarker expression of CTCs in patients with metastatic gastric cancer

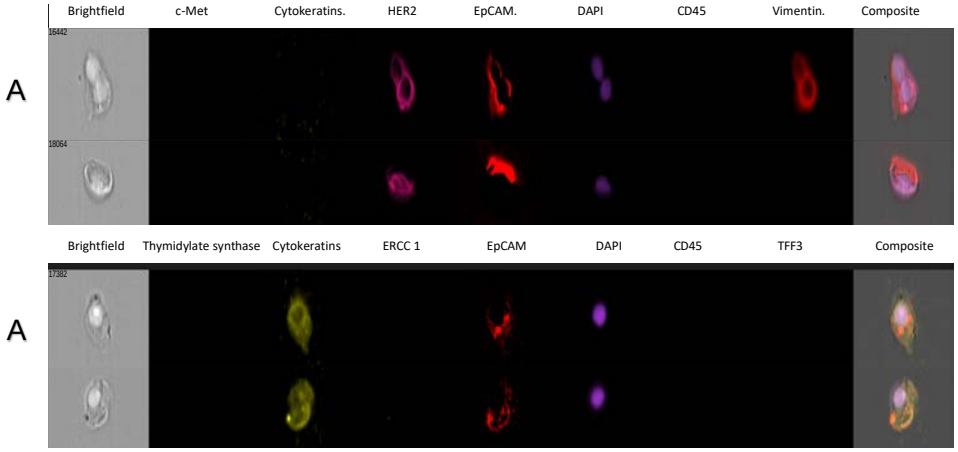
The majority of the CTCs in patients with metastatic gastric cancer expressed cytokeratins. Both patients P11 and P10 had some CTCs which did not express any of the biomarkers in the panels. Primary tumour was HER2 negative in patients P11, P10 and P9. None of the patients received trastuzumab. However, patient P11 had a population of CTCs which expressed cytokeratins and HER2.

6.2.3 Characterisation of circulating tumour cells in the blood following palliative chemotherapy

Patient P3 had a locally advanced junctional oesophagogastric adenocarcinoma with pulmonary metastases. The primary tumour was HER2 positive and the patient received six cycles of HCX (trastzumab, capecitabine, cisplatin). Blood samples were obtained at prior to start of the treatment and at 8 weeks following completion of treatment. A CT scan performed following completion of treatment showed that the primary tumour had progressed but the pulmonary metastases had reduced in size. Figure 6.2.3.1 illustrates images of CTCs in the blood before and after completion of the chemotherapy regime.

P3 – Metastatic junctional oesophagogastric adenocarcinoma with pulmonary metastases

CTCs in pre-treatment sample



*Panel A sample lost and not analysed

Palliative chemotherapy regimen – Trastuzumab, cisplatin, capecitabine (HCX)

CTCs in post treatment sample

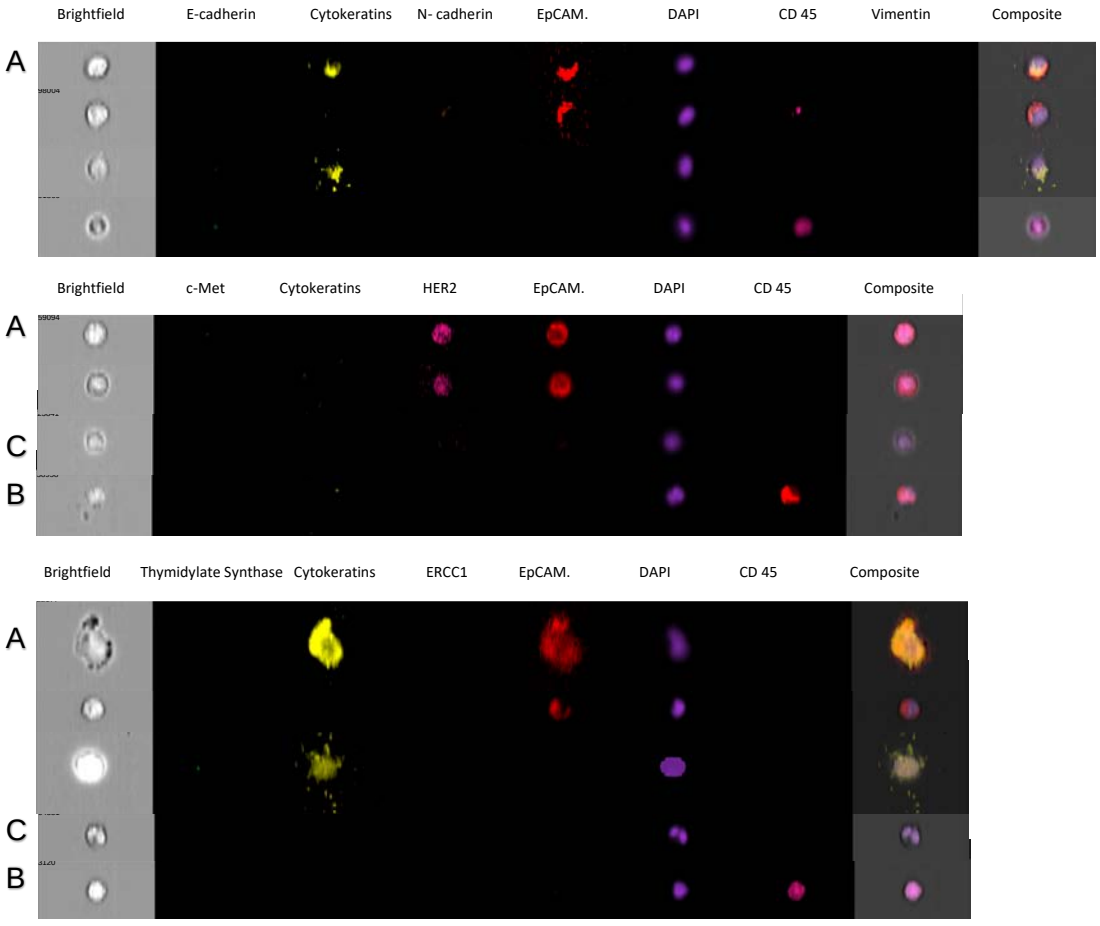


Figure 6.2.3.1– Images of CTCs and white blood cells in the blood sample of patient P3, pre and post palliative chemotherapy

A: Biomarker positive CTCs in the pre and post treatment sample. B: white blood cells in the pre and post treatment sample. C: Biomarker negative CTCs in the pre and post treatment sample

In the pre-operative sample, a total of 62 CTCs per 7.5 ml of blood were detected. There was one population of CTCs identified in panel C which expressed EpCAM and cytokeratins and accounted for 29 CTCs per 7.5 ml of blood. In panel B, there was one population of CTCs which expressed HER2 and EpCAM and accounted for 33 CTCs. Data from panel A was lost during the flow cytometry analysis. Following chemotherapy, a CT scan was performed to assess the response of the cancer to the chemotherapy regimen. This revealed that the primary tumour did had not responded to the chemotherapeutic regimen and had increased in size. However, the pulmonary metastases reduced in size. There were five different populations of CTCs present in the blood sample collected post chemotherapy treatment. The total number of biomarker positive CTCs increased from 62 to 79 CTCs per 7.5 ml of blood. Two populations of CTCs remained. CTCs which expressed EpCAM and cytokeratins decreased in number from 29 to 14 CTCs per 7.5 ml of blood. CTCs which expressed EpCAM and HER2 increased from 33 to 43 CTCs per 7.5 ml of blood. There were three other population of CTCs present. The first population expressed cytokeratins alone accounted for 16 CTCs per 7.5 ml of blood. The second population expressed EpCAM alone and accounted for 16 CTCs per 7.5 ml of blood. One can hypothesise that this population of CTCs based on their size, shape and nuclear density arise from the equivalent population of CTCs which express EpCAM and HER2 detected in panel B. There were a total of 72 biomarker negative CTCs per 7.5 ml of blood present in the postoperative sample.

6.2.5 Characterisation of tumour cells in exudates in patients with metastatic oesophagogastric cancer

The presence of tumour cells which have infiltrated the lining of the peritoneal and pleural cavities results in the production of proteinaceous fluid. Fluid within the peritoneal cavity is referred to as ascites. The accumulation of fluid within the cavities can cause symptoms of abdominal pain and bloating. Symptomatic relief can be obtained in the short term by draining the fluid. Long term drains can be left in the cavities to allow repeat drainage. Exudates were

obtained in four patients who underwent symptomatic drainage of ascites as illustrated in Table 6.2.1.1. Table 6.2.5.1 illustrates details of the exudates obtained from the four patients in the study.

Table 6.2.5.1 Demographics of patients recruited into the study undergoing palliative treatment for oesophagogastric cancer.

Patient	Tumour site	Site of metastases	Exudate Volume (mls)	Exudate volume for high resolution imaging flow cytometer (mls)	Exudate volume for Culture (mls)
P10	Gastric adenocarcinoma	Peritoneal disease – ascites	250	100	150
JR	Recurrent Gastric cancer – STG 2017	Peritoneal disease – ascites	500	200	300
SH1	Oesophagogastric junctional adenocarcinoma	Peritoneal disease – ascites	300	100	200
BB1	Oesophagogastric junctional adenocarcinoma	Peritoneal disease – ascites	300	100	200

The volume of ascites and the composition of the ascitic fluid varied between patients. Patient JR had a large volume of ascites drained at the time of presentation. However, the fluid contained a large amount of cellular debris which required filtration prior to centrifugation. There was a wide variation in the cellular composition of the fluid between samples. The cellular component of the ascitic fluid was isolated and labelled with the antibodies from the three biomarker panels used to label CTCs. The method for isolation of the tumour cells from the ascites is found in Chapter 2, Section 2.2.2. Figure 6.2.5.2 illustrates images of CTCs in the blood and tumour cells in the ascites of patient P10.

P10 – Proximal gastric cancer – T4N2M1– Peritoneal disease with ascites

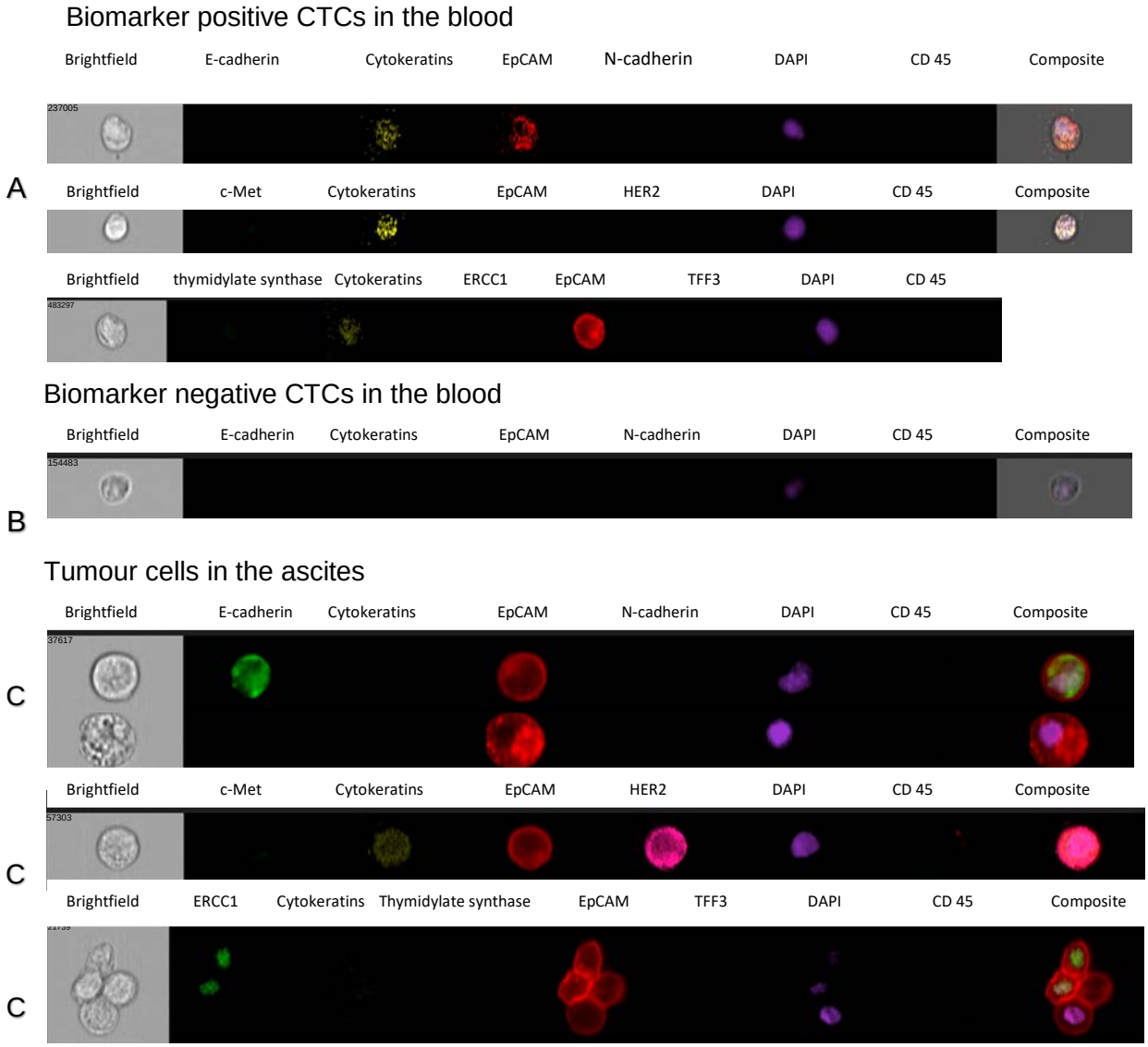


Figure 6.2.5.2 – Images of CTCs in the blood and tumour cells in the ascites in patient P10 – Proximal gastric cancer with peritoneal disease and ascites

A: Biomarker positive CTCs in the blood sample B: Biomarker negative CTCs in the blood sample C: tumour cells in the ascites

There were three populations of CTCs present in the blood in patient P10. One population of CTCs expressed EpCAM, the second expressed EpCAM and cytokeratins and the third population of CTCs did not express any of the biomarkers in the panels. The ascites had heterogenous population of cells in the blood which expressed a number of different biomarkers. The primary tumour was HER2 negative but the ascites contained tumour cells

which expressed HER2, EpCAM, E-cadherin and cytokeratins. In addition, there was a population of CTCs which expressed thymidylate synthase and EpCAM. These tumour cells were present in clusters. There were no biomarker negative tumour cells in the ascites.

Figure 6.2.5.3 illustrates images of CTCs and tumour cells in the ascites in patient JR.

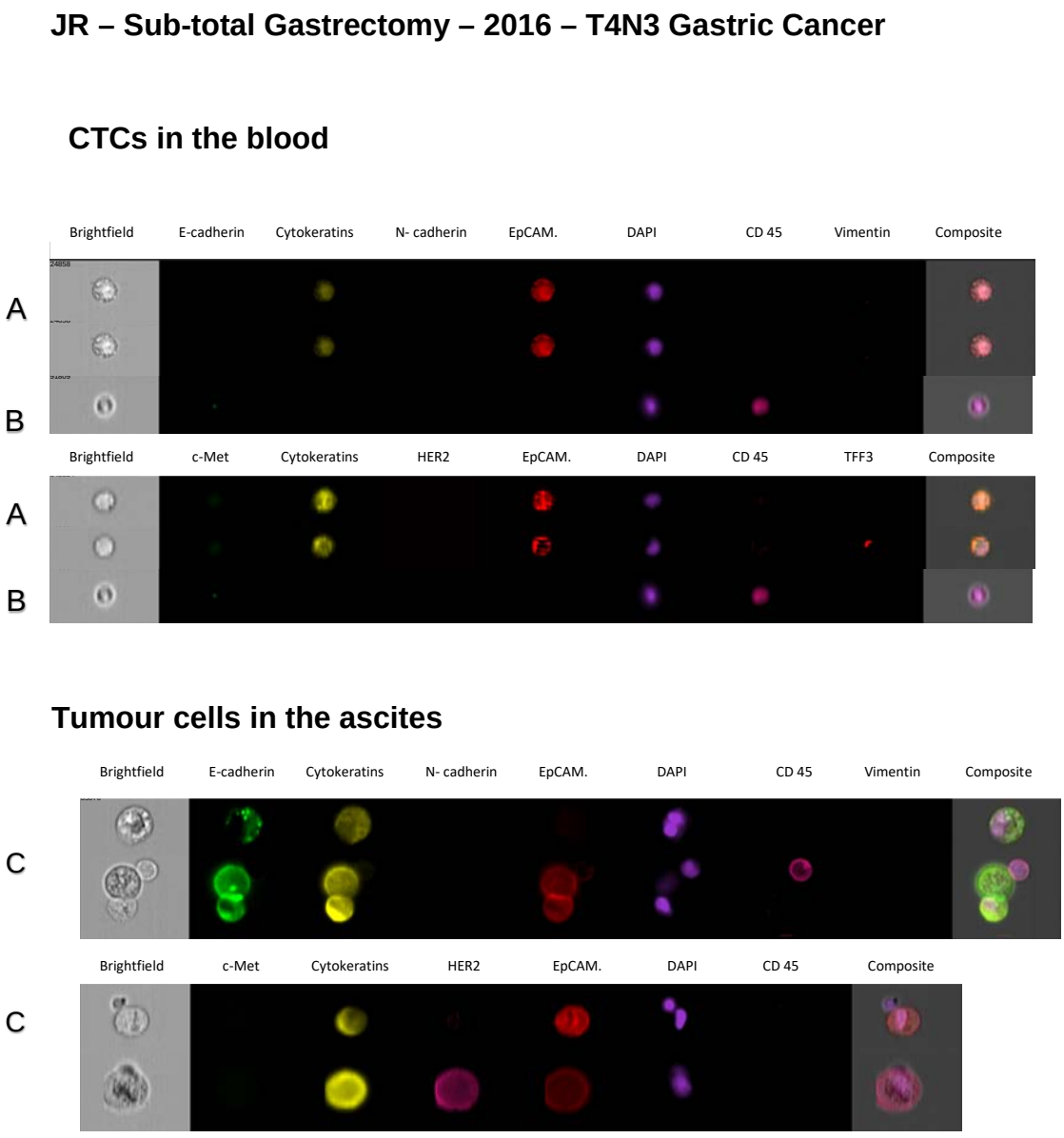


Figure 6.2.5.3 – Images of CTCs in the blood and tumour cells in the ascites in patient JR – Sub-total gastrectomy T4N3 adenocarcinoma, Local and peritoneal recurrence of disease with ascites.

A: CTCs in the blood sample B: white blood cells in the blood sample C: tumour cells in the ascites

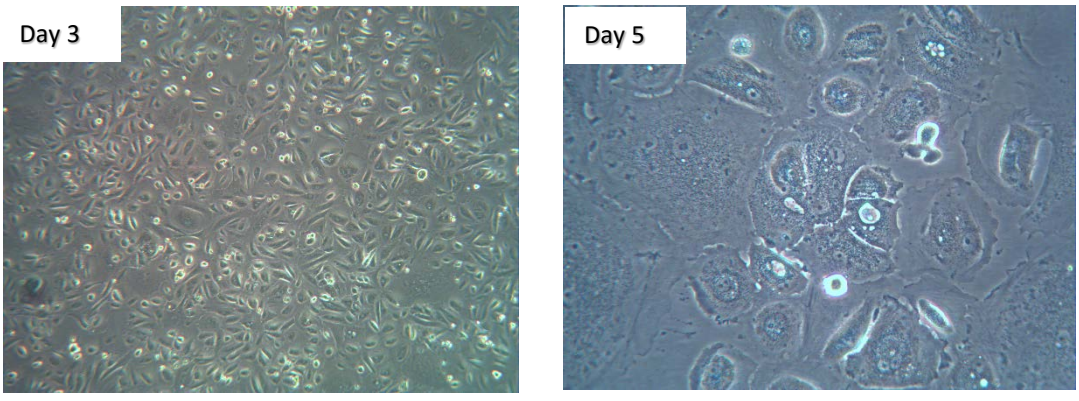
Patient JR had only one population of CTCs in the blood, which expressed EpCAM and

cytokeratins. There were multiple populations of tumour cells in the ascites in the two panels analysed. In panel A, two populations of tumour cells were detected. One population of tumour cells expressed E-cadherin, cytokeratins and EpCAM and the other expressed E-cadherin and cytokeratins. In panel B, there were two population of tumour cells detected, one expressed cytokeratins and EpCAM and the other expressed cytokeratins, EpCAM and HER2.

The tumour cells in the ascites were heterogenous in their morphology and biomarker expression. There was variation in the size of the tumour cells and their shape and in biomarker expression within the populations of tumour cells present.

6.2.6 Culture of tumour cells from exudates in patients with metastatic oesophagogastric cancer

Ascitic fluid was collected in sterile conditions into a sterile flask. The fluid was passed through a 70 micron filter to exclude the cellular debris. The suspension post filtration was placed into the culture medium listed in table 2.2.2.1 in Chapter 2. Details of the cell culture are present in Chapter 2, Section 2.2.2. Tumour cells isolated from the exudate from patient P10 were cultured as per the protocol The cells were imaged in the flasks with light microscopy on day 3, 5 and at day 7. Figure 6.2.6.1 illustrates images of tumour cells from patient P10 cultured in DMEM + 20% FBS + 1% Pencillin-Streptomycin (10 000 U/ml) obtained at day 3 and day 5.



40 x objective

80 x objective

Figure 6.2.6.1 – Images of tumour cells in culture from the ascites from patient P10

A: Images of tumour cells in culture at day 3 B: Images of tumour cells in culture at day 5

Figure 6.2.6.1 illustrates the growth and proliferation of the tumour cells in culture. The cells were trypsinised at day 7. The cell suspension post trypsinisation from one flask was centrifuged and labelled with the antibodies from the three biomarker panels. The cell suspension from the second flask was divided into two samples and placed into culture. Figure 6.2.6.2 illustrates images of tumour cells from patient P10 following trypsiniation captured on the high resolution imaging flow cytometer.

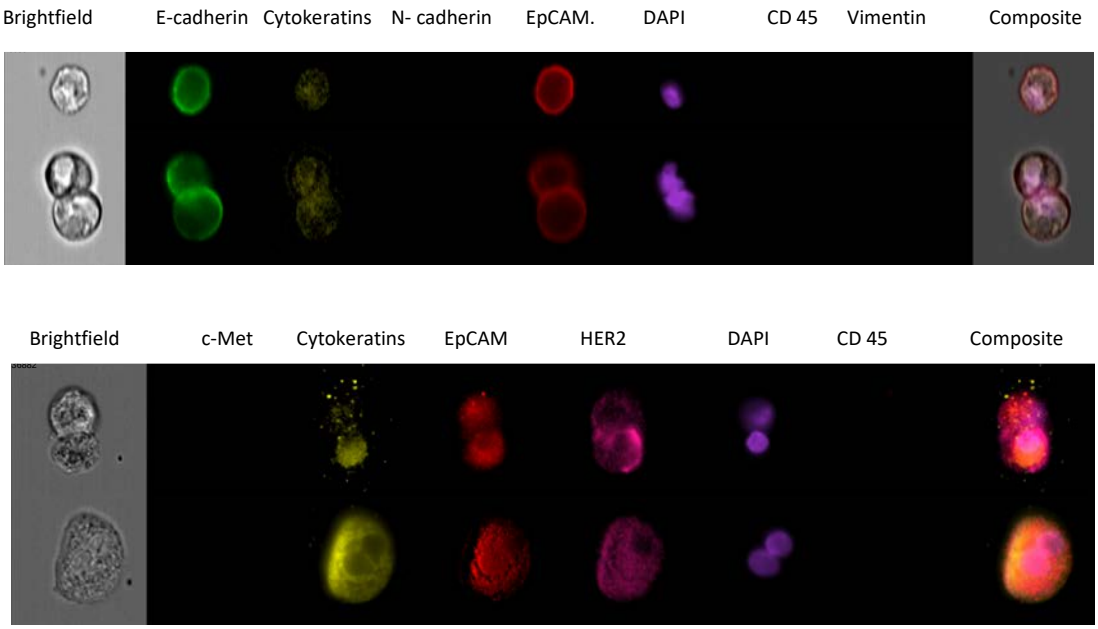


Figure 6.2.6.2 – Images of tumour cells from the ascites in patient P10 following culture and trypsinisation on the high resolution imaging flow cytometer

A: Images of tumour cells in culture at day 3 B: Images of tumour cells in culture at day 5

The biomarker expression of tumour cells in the ascites pre and post culture was similar as shown in Figure 6.2.5.2. Tumour cells detected prior to culture expressed EpCAM and E-cadherin (panel A) and also EpCAM, cytokeratins and HER2 (panel B). Post culture, the tumour cells which express EpCAM, cytokeratins and HER2 remain. Tumour cells which expressed EpCAM and E-cadherin in the ascites pre culture in panel A, now expressed cytokeratins as well. The fact that in both panel A and B, the cells expressed EpCAM and cytokeratins, one could hypothesise that the cells detected post culture may all be part of the same population.

In all four patients there was evidence of viable tumour cells based on the evaluation of cell proliferation using light microscopy up to passage two.

6.3 Discussion

There was a wide variation in the number of CTCs in patients with advanced oesophagogastric cancer. Patients with locally advanced disease with the presence of distant metastases had a significantly higher number of CTCs than patients with locally advanced cancer that was not amenable to curative resection (p=0.021). Of note, there was no significant difference in the number of CTCs in patients who had locally advanced disease with nodal metastases outside the field of curative resection compared to patients undergoing curative surgery alone with locally advanced disease (p=0.657).

There is concordance between the biomarker expression of the primary tumour and the CTCs in the blood. All bar one of the patients in whom the primary tumour was HER2 positive had CTCs that expressed HER2. Patient P11 had CTCs in the blood which expressed HER2 but the primary tumour was reported negative for HER2 expression and amplification.

A similar observation was made on the measurements of CTCs in patient P3 pre and post chemotherapy treatment. The primary tumour in patient P3 expressed HER2. CTCs that expressed HER2 were present in the blood prior to treatment with palliative chemotherapy. The patient received six cycles of HCX chemotherapy. Following treatment, a repeat CT showed that the primary tumour increased in size but the pulmonary metastases reduced in size. Blood samples post chemotherapy treatment revealed that CTCs which expressed EpCAM and HER2 remained and increased in number. Another population of CTCs which expressed EpCAM and cytokeratins decreased in number. In addition, three new population of CTCs were now present. Of note, one of these populations of cell did not express any of the biomarkers in the panels. These findings suggest the presence of multiple clonal populations of cells within the primary tumour with different biomarker expressions. This suggests that chemotherapeutic regimens target only a select clonal population of cells within the primary tumour and at sites of metastases. Tumour cells arising from a different clone which are resistant to the chemotherapy regime proliferate as a consequence. This would explain the different responses to the chemotherapy between the primary tumour and the pulmonary

metastases in patient P3. One can hypothesise the origin of the CTCs in the blood based on the change in the number of CTCs present between the pre and post treatment samples. The reduction in the size of the pulmonary metastases following treatment correlated with the reduction in the total number of CTCs which expressed EpCAM and cytokeratins alone. This could indicate the origin of the CTCs from the pulmonary metastases. In addition, the poor response of the primary tumour to the chemotherapy regime, correlates with the increase in the number of CTCs which expressed EpCAM and HER2 which is consistent with some of these cells originating from the primary tumour. There were 72 CTCs per 7.5 ml of blood post treatment which did not express any of the biomarkers present in the panels. The origin of these cells is unknown.

The impact of chemotherapy on tumour evolution remains unknown. Characterisation of the biomarker expression and morphology of CTCs would aid in monitoring the response to the chemotherapy of the primary tumour and metastatic sites. Future work would focus upon isolating individual populations of CTCs based upon biomarker expression and then sequence the transcriptome. This would help identify therapeutic targets for the chemotherapy agents with the future potential for individualised cancer treatment.

CTC clusters are hypothesised to have a longer half life in the blood compared to single cell CTCs (160). The formation of clusters is thought to provide protection from cells within the immune system and provide protection against environmental and oxidative stresses (160, 161). CTC clusters are thought to have enhanced metastatic potential in comparison to single CTCs which is attributed to multiple factors. CTC clusters can contain a heterogenous population of CTCs that express epithelial and/or mesenchymal markers (160). This heterogeneity may facilitate the dissemination and proliferation of the cells from the primary tumour to distant sites. In this present study, CTC clusters were present in five out of the twelve patients with advanced oesophagogastric malignancy. The clusters identified contained cells with a homogenous population of CTCs.

The successful culture of tumour cells from the ascites provides a template for developing suitable culture conditions and a protocol to culture CTCs from the blood. However, the numbers of tumour cells in ascites are far greater than the numbers of CTCs in blood and they

express different biomarkers. The challenge is to develop a protocol to isolate a sufficiently large number of CTCs from the blood to allow it to be placed into culture.

Chapter 7: Discussion

7.1 Assessment of Achievements

7.1.1 Development of a reliable assay for detecting CTCs in the blood

The first aim of the study was to develop a reliable assay to detect and characterise CTCs in the blood in patients undergoing curative and palliative treatment for oesophagogastric cancer. Refinements to the previous protocol for the analysis of CTCs in the blood after immunomagnetic separation of white blood cells were made which led to an improvement in the enrichment and recovery of cells from the blood. In addition, time taken for processing individual samples was reduced from 5 to 3 hours.

Two other methods of isolation and enrichment of CTCs from blood were evaluated. In EasySep™ Direct Human CTC Enrichment, red and white bloods cells are removed by immunomagentic separation during the enrichment of CTCs from blood. The cells isolated by this method could be placed directly into culture. Althought the method has potential, the enrichment and recovery of cells was poor and consequently clinical application for the evaluation of CTCs is limited. Parsortix^{TM} offered a simplified process to isolate CTCs directly from blood based on their size. The major limitation of the process was the difficulty in the positive identification of CTCs following any enrichment. Labelling the cells captured in the cassette post enrichment with antibodies and subsequent imaging was challenging because the small number numbers of cells recovered meant that they would be lost during any procedures involved in their analysis.

In the present study, a protocol was developed to isolate cells by fluorescence activated cell sorting based upon their biomarker expression. Oesophageal carcinoma cells were isolated successfully from blood for imaging by confocal microscopy and high resolution imaging flow cytometry, and were placed into culture.

7.1.2 Development of a reliable assay for detecting DTCs in the bone marrow of excised rib

The protocol for isolation and enrichment of DTCs from bone marrow was as adaption of the method used to analyse CTCs. Enrichment and recovery of DTCs from the bone marrow in the

initial experiments was approximately 50% based on the optimization experiments. DTCs were detected in the bone marrow of all patients undergoing curative treatment open oesophagectomy for oesophageal adenocarcinoma.

7.1.3 Characterisation of the heterogeneity within the CTCs in the blood and DTCs in the bone marrow

The high resolution imaging flow cytometer had multiple channels which could detect in principle up to 10 different biomarkers and characterise cells based on their size and morphology. A wide range of biomarkers were incorporated into the study which helped to identify and characterise the heterogeneity within the CTC population. CTCs were detected in all patients undergoing curative and palliative treatment for oesophageal cancer. There was variation in the biomarker expression within the CTC populations isolated in both the curative and palliative treatment cohort of patients. In the curative treatment cohort, less than 50% of CTCs detected expressed both EpCAM and cytokeratins. In addition, 50% of patients in the curative cohort had a heterogeneous population of CTCs which was associated with a poor prognosis. The study identified a population of cells that did not express any of the biomarkers included in the panel but had similar morphological characteristics to CTCs. These biomarker negative CTCs were present in patients undergoing curative and in patients undergoing palliative treatment. In the curative treatment cohort, the biomarker negative cells were present both in patients with early cancer and in patients with locally advanced oesophageal cancer who had received peri-operative chemotherapy.

7.1.4 Prognostic significance of CTCs in the blood and DTCs in the bone marrow

In this study, two year disease free survival in patients Stage III and IV cancer who underwent surgery alone was 25%. In those who received peri-operative chemotherapy and surgery, two year disease free survival was 48%. Fourteen out of the twenty-three patients who received peri-operative chemotherapy and surgery developed recurrence within the two year follow up period, median time to recurrence was 21 months. For patients staged clinically with Stage III and IV disease, two year disease free survival was 38% and median time to recurrence was 20 months.

Patients with 100 or more CTCs per 7.5 ml of blood in the postoperative sample had a significant shorter two year disease free survival (p=0.012) than patients with fewer than 100 CTCs per 7.5 ml of blood. In addition, patients with a heterogeneous population of CTCs in the pre-operative sample based on biomarker expression had a significant shorter two year disease free survival (p=0.043).

The presence of lymphovascular invasion and poor response to peri-operative chemotherapy based on the tumour regression grade are recognized poor prognostic factors in patients undergoing curative treatment for oesophagogastric cancer.

The number of DTCs and CTCs in the postoperative samples were significantly higher in patients with the presence of lymphovascular invasion (p=0.051) (p=0.010). This observation suggests that in patients with evidence of lymphovascular invasion in the primary tumour, there is dissemination of tumour cells from the primary tumour to distant sites. This could explain the rise in the postoperative CTCs in patients with evidence of lymphovascular invasion in primary tumour despite the resection of the primary tumour (p=0.007). This suggests that the origin of the CTCs in the postoperative sample are from a distant metastatic site.

There was a rise in the number of CTCs between the pre and postoperative samples in patients who had a poor response to peri-operative chemotherapy based upon TRG scores of 4 or 5. In contrast, the total number of CTCs fell between the pre and postoperative samples in patients who had a good response to chemotherapy and had a TRG score of 3 or less. However, there was no significant difference between the total number of postoperative CTCs in patients who had a poor response to chemotherapy and those who had a good response to chemotherapy. If CTCs originated from the primary tumour, one might expect CTCs in the blood to fall following resection of the primary tumour. The rise in the number of CTCs in the postoperative sample in patients would be consistent with the source of CTCs being a site other than the primary tumour. The bone marrow is a potential reservoir for tumour cells following resection and could be the source of the disseminated tumour cells that lead to early relapse after resection of the primary tumour in patients who do not respond to chemotherapy. All twelve patients who had a poor response to chemotherapy based on TRG 4 or 5 who relapsed had the at least one population of cells in the bone marrow and the postoperative blood sample that expressed the same biomarkers.

In patients treated with palliative intent, the number of CTCs correlated with the tumour burden. Patient with the presence of distant metastases and ascites had a significantly higher number of CTCs compared to patients with locally advanced cancer that was not amenable to curative surgical resection (p=0.021). Sequential measurements of CTCs in one patient who received palliative chemotherapy revealed a difference in heterogeneity in biomarker expression of CTCs identified before and after chemotherapy treatment. In patient P3, the primary tumour was HER2 positive and the patient received HCX chemotherapy regimen. There was progression of the primary tumour burden based upon a CT scan which following chemotherapy. However, there was regression of the pulmonary metastases on the CT scan indicating a response of these metastases to the chemotherapy. There was a rise in the total number of CTCs between the pre and post treatment sample, 62 to 151 CTC per 7.5 ml of blood. There was an increase in the number of CTCs that expressed EpCAM and HER2 but a fall in the number of CTCs that expressed EpCAM and cytokeratins. In addition, there was a new population of CTCs which expressed cytokeratins and a population of biomarker negative CTCs. These observations indicate the presence of heterogeneous population of tumour cells within the primary tumour and distant metastatic site. The difference in response of the primary tumour and distant metastatic sites is reflected by the change in the total number of CTCs and the different populations of CTCs based on biomarker expression present in the circulation before and after treatment.

In the future, characterising the biomarker expression in the CTCs could help identify therapeutic targets for chemotherapy regimens. Sequential monitoring of CTCs will provide the ability to monitor response to treatment and modify therapeutic regimens during treatment to target tumour cells that have not been eliminated by the initial therapy.

7.2 Comparison between outcomes of present studies with those reported in the literature

7.2.1 CTC detection rates in oesophageal adenocarcinoma

Four studies have detected CTCs in patients undergoing curative treatment for oesophageal adenocarcinoma. Two studies reported CTCs detected based upon the combined expression of two epithelial makers combined, EpCAM and cytokeratins as per the CellSearch^{R} test (128,

129). Cells were classified as CTCs only if they expressed both EpCAM and cytokeratins. In the study by Reeh et al, 20% (14/68) patients with oesophageal adenocarcinoma had CTCs (129). This was similar to the findings of the study by Konczella et al, 20% (15/76) patients had CTCs (128). In both studies small numbers of CTCs were detected and the presence of one or more CTCs was deemed significant. Both studies concluded that the presence of CTCs was associated with shorter relapse free and overall survival. Two other studies, enriched CTCs from the blood using a size based filter. Cells greater than 8 microns in diameter were isolated and stained with nuclear and cytoplasmic markers. Detection rate of CTCs were higher in these studies. Bobek et al reported detection of CTCs in 75% (15/20) patients (130). Kuvendjiska et al, reported detection of CTCs in 60% (12/20) of patients who underwent curative treatment for oesophageal adenocarcinoma (132).

In the present study, CTCs were present in all patients undergoing curative treatment for oesophageal adenocarcinoma. The proportion of CTCs which expressed both EpCAM and cytokeratins varied depending upon the clinical stage of disease at the time of surgery and between the pre and postoperative blood samples. In patients with early oesophageal cancer, CTCs which expressed both EpCAM and cytokeratins were present in only 44% (4/9) patients in the pre-operative sample and 33% (3/9) patients in the postoperative sample. In patients who received peri-operative chemotherapy and surgery, CTCs which expressed both EpCAM and cytokeratins were present in 45% (11/24) patients in the preoperative sample and in 63% (15/24) patients in the postoperative sample. Overall, detection rate of CTCs which expressed both EpCAM and cytokeratins alone in patients undergoing curative treatment was 49% in the pre-operative sample and 60% in the postoperative sample. This is higher than in previous studies which only detected CTCs using this criteria in 20% of patients.

The use of multiple biomarkers combined with the high resolution imaging flow cytometer enables the detection and characterisation of multiple populations of CTCs in the circulation. This could explain the overall higher detection rates of CTCs in this study when compared to the literature. In this present study, heterogeneous populations of CTCs were detected in the pre-operative blood sample in 24 patients and in the postoperative blood sample in 23 patients. The presence of heterogeneous population in the preoperative sample was associated with shorter disease free survival ($p=0.043$). In addition, this present study highlights the fact that there are CTCs in the blood which do not express EpCAM.

A previous study reported the presence of HER2 expression in CTCs in patients with potentially curative gastric cancer in whom the primary tumour was HER2 negative. Subsequent treatment with trastuzumab in patients where the primary tumour did not express HER2 but the CTCs expressed HER2 revealed a comparable response to the findings of the TOGA trial (162). In the present study, the biomarkers most frequently detected in addition to EpCAM and cytokeratins in the CTCs, were c-Met and HER2. CTCs which expressed HER2 were present in four patients in the pre and seven patients in the postoperative sample.

7.2.2 Evidence that CTCs have undergone EMT

CTCs are thought to arise from the primary tumour and undergo EMT to facilitate their dissemination to distant sites within the body (74, 96, 98). It is hypothesised that during this process, there is a change in the phenotype of the cell with subsequent loss of cell junctions and apical-basal polarity. The cells lose their expression of epithelial biomarkers, eg EpCAM and cytokeratins when they undergo EMT and enter the circulation (163). As a consequence, methods which rely on detection of CTCs based on epithelial biomarker expression do not identify this population of CTCs. These cells are also hypothesised to have the greatest malignant potential due to their resistance to anoikis and chemo and radiotherapy (74, 164). In this study, a proportion of cells detected did not express any of the biomarkers analysed but had the morphological characteristics of CTCs based on their size and nuclear density. The biomarker negative CTCs were present in 19 out of 37 patients undergoing curative resection for oesophagogastric cancer. However, the number of the biomarker negative CTCs in patients who received peri-operative chemotherapy was higher than in patients with early oesophagogastric cancer in both the pre and postoperative samples ($p=0.023$)($p=0.034$). The findings suggest that this population of cells has undergone EMT and thus do not express the epithelial biomarkers present in the study. Further characterisation of the cells with additional mesenchymal biomarkers combined with molecular analysis by RNA sequencing will be interesting and will aid in identifying the origin of these cells.

7.2.3 Prognostic significance of CTCs in patients undergoing curative treatment for oesophageal adenocarcinoma

The presence of CTCs in the blood has been associated with poor prognosis in patients undergoing curative treatment for oesophagogastric cancer. The threshold of the number of CTCs required for significance varied between studies. In the previous two studies, the presence of one or more CTCs was associated with a significant decrease in overall and disease free survival (128, 129)(p=0.001). In this study, the presence of a heterogenous population of CTCs in the pre-operative sample and/or the presence of 100 or more CTCs per 7.5 ml of blood in the postoperative sample was associated with a significant decrease in disease free survival (p=0.043)(p=0.001). A meta-analysis of the prognostic significance of CTCs in oesophageal cancer concluded that CTC status correlated with the clinical TNM and T stage but not with lymph node metastases (165). In the present study, there was no significant correlation between the CTC status and clinical TNM stage (p=0.087).

7.2.4 Disseminated tumour cells in the bone marrow in patients with oesophageal adenocarcinoma

DTC detection rates in the bone marrow from the rib from previous studies varied between 45-88% in patients undergoing curative treatment for oesophagogastric cancer (108, 112, 114, 116, 117). The variation in the detection rates was related to the different methods of isolating DTCs from the bone marrow. Two long term follow up studies came to different conclusion regarding the prognostic significance of DTCs. Konczella et al, reported the detection of DTCs from the iliac crest in 17% (13/76) patients and CTCs in the blood in 20% (15/76) patients with non-metastatic oesophageal cancer(128). In addition, CTCs and DTCs were detected together in only 3 patients in the study. The study concluded that the presence of one or more CTCs were an independent prognostic factor for survival. DTCs were not of prognostic significance.

In the present study, DTCs were present in all patients undergoing curative resection for oesophagogastric cancer. There was no significant difference in the number of DTCs in patients with early and locally advanced oesophagogastric cancer (p=0.992). Previous studies have commented upon the variation in the biomarkers expression between the primary tumour and DTCs in the lymph node and bone marrow (146). Dreimel et al, demonstrated

variation in the expression of epithelial biomarkers between the primary tumour and DTCs in the bone marrow (163). In 64% of patients, the DTCs in the bone marrow did not express EpCAM despite the primary tumour being EpCAM positive. Molecular analysis of individual DTCs in the bone marrow had demonstrated poor prognosis in patients in whom the presence of HER2 gain was detected in the DTCs and not in the primary tumour. There was a heterogeneous population of DTCs present in the bone marrow from the rib in this study. DTCs that expressed novel biomarkers were present in 70% (23/33) patients, of which 9 patients had DTCS that expressed HER2. Correlation of these findings with the biomarker expression in the primary tumour will be of interest.

7.3 Comparison with literature on the significance of circulating tumour cells in patients with metastatic oesophageal adenocarcinoma

CTCs have been reported to be independent predictors of progression free survival in patients with metastatic solid organ tumours (65, 66, 165). The presence of 5 or more CTC was associated with a significant reduction in progression free survival in patients with metastatic breast cancer. Previous studies have reported the presence of one or more CTCs in patients with metastatic oesophagogastric cancer is associated with a reduction progression free survival (165, 166). Detection rates of CTCs in these studies varied between 21 to 44% (165, 166).

In the present study, the number of CTCs detected in this group of patients varied between 40 to 1988 CTCS per 7.5 ml of blood. Eight out of twelve patients in the study had evidence of distant metastatic disease, and two of the patients had liver metastases and ascites. This group of patients had significantly higher number of CTCs than the patients who had locally advanced disease not amenable to curative resection (0.021). Of interest, there was no significant difference in the number of CTCs in patients with locally advanced cancer in whom the disease was not amenable to curative resection compared to those who underwent surgery for locally advanced cancer (p=0.950). Both observations indicate that the CTCs play a role in the dissemination of cells from the primary tumour to distant sites. The findings in the study supports the current literature which indicate the presence of CTCs is associated with poor prognosis.

7.4 Limitations

As the study progressed, improvements in efficiency in the collection, storage and processing of samples led to improvements of enrichment and recovery of CTCs in the blood and DTCs in the bone marrow. The original protocol used in the processing of early patient samples which gave a lower recovery of CTCs as shown in Chapter 3. As a consequence, the number of CTCs and DTCs detected in early patient samples could be an underestimate of the total numbers present.

Cells were visualized on the high resolution imaging flow cytometer and images reviewed by a single individual for some of the patients. Positive identification of CTCs and DTCs in this study was based on both morphological characteristics and biomarker expression. Although the classification criteria were strict, for some of the patients, the images were not reviewed by another individual to confirm the findings. Thus, one potential limitation in the study is that of observer bias.

One of the strengths of the study was to incorporate a wider range of biomarkers to characterize the heterogeneity. As a consequence, three different panels of antibodies were analysed. Each panel incorporated EpCAM and cytokeratins but different combination of novel biomarkers were present. Therefore, HER 2 and c-Met expression was only assessed in 1/3 of the cells analysed in each sample. A limitation of the study is that the number of CTCs expressing the novel biomarkers may be an underestimate of the total number present.

7.5 Summary of Novel Findings

In the present study, all patients who were diagnosed with oesophagogastric cancer had evidence of CTCs present in the blood. The detection rate can be attributed to the ability of the high resolution imaging flow cytometer to image and characterise individual cells based on their morphology and biomarker expression. The incorporation of a wide range of biomarkers helped characterize the heterogeneity of biomarker expression within the CTC and DTC population. In over 50% of patients who underwent curative treatment, there were two or more populations of CTCs based on biomarker expression were present. In the majority of

patients, CTCs would not have been detected if the CellSearch^{R} method was utilised which only identifies a CTC if it expresses both EpCAM and cytokeratins. This finding could explain the higher number of CTCs detected in this study compared to previous studies in the reported literature.

Our study identified biomarker negative CTCs which were morphologically similar to the CTCs that expressed biomarkers present in the panels. In some patients, these cells accounted for the majority of the CTCs present in the blood. The origin and the function of these cells remains unknown and warrants further study.

In the curative cohort, the presence of 100 or more CTCs per 7.5 ml of blood in the postoperative sample was associated with poor prognosis. In addition, the number of CTCs and DTCs were higher in patients who had a poor response to chemotherapy based on the TRG grade. In the palliative cohort, the number of CTCs in the blood correlated with the burden of the disease. Patients who had evidence of locally-advanced disease had significantly lower numbers of CTCs than patients with extensive metastatic disease.

7.6 Future work

The present study highlights the clinical potential of CTCs and DTCs in the blood and bone marrow in patients undergoing curative treatment for oesophagogastric cancer. There are multiple avenues for future research. The role of CTCs in the evolution of the primary tumour can be evaluated further by sequential measurements of CTCs at defined intervals during the treatment pathway. Detection of therapeutic biomarkers, eg HER2 in CTCs and in the primary tumour could provide evidence for the use of trastuzumab in patients undergoing curative treatment for oesophageal cancer. Evaluating the change in the CTC populations and the biomarker expression during treatment could provide information regarding tumour biology and the molecular mechanisms of tumour cell dissemination.

Different populations of CTCs could be isolated from blood by fluorescence activated cell sorting based on biomarker expression for subsequent genomic and transcriptomic analyses and to plate CTCs into culture. Successful culture of CTCs ex vivo could help develop new therapeutic regimens. New chemotherapy agents could be evaluated ex vivo with using CTCs

grown in culture. The overall aim would be the incorporation of the genomic and transcriptomic information from the primary tumour and CTCs into the current staging protocol. Improved prognostic information derived from the staging protocol can help counsel patients for the available treatment modalities.

In the present study and literature, 50% of patients with locally advanced cancer relapse within two years following surgery. At present, there are no established biomarkers to predict recurrence in oesophagogastric cancer or defined follow up protocols for patients following curative resection for oesophagogastric cancer. Patients are reviewed in outpatient clinics and imaging is requested if they develop symptoms or signs of relapse. At this point, the tumour burden may be too advanced for successful treatment. Measurement of CTCs in the postoperative period following surgery could help identify recurrence earlier in patients before the patients develop symptoms. Combined with radiological imaging of these patients, sites of relapse could be identified earlier at a stage when they may be amenable for radical treatment, eg isolated pulmonary or liver metastases for surgical resection. This surveillance could potentially improve overall survival and hence the benefit of curative treatment in patients.

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Appendix A:

Health Research Authority

Mr Anantha Madhavan

Northern Institute for Cancer Research Paul O'Gorman Building

Email: hra.approval@nhs.net

Newcastle Upon Tyne

NE2 4HH

19 September 2016 Dear Mr Madhavan

Letter of HRA Approval

Study title:	**Does the presence of bone marrow or circulating tumour cells predict early tumour recurrence in patients with oesophagogastric cancer?**
IRAS project ID:	**185350**
REC reference:	**16/NE/0241**
Sponsor	**Newcastle upon tyne NHS foundation trust**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Your IRAS project ID is **185350**. Please quote this on all correspondence. Yours sincerely

Dr Claire Cole Senior Assessor

Appendix B:

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

CONSENT FORM

Study ID Number:

Researchers:
Professor SM Griffin Consultant Oesophagogastric Cancer Surgeon
Mr Anantha Madhavan Surgical Research Fellow

Analysis of upper gastrointestinal cancer tissue, blood and exudates

Please initial the box in response to each statement

I confirm that I have read and understood the information sheet dated August 2016 (Version 2) for the above study. ☐

I allow access to my medical records by the investigators in the study. ☐
I understand that the relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Newcastle upon Tyne NHS Foundation Trust and Regulatory bodies, where it is relevant to my taking part in this research.

I have had the opportunity to consider the information, ask questions and have these answered satisfactorily ☐

I agree to participate in the study and that blood, and tissue and exudates that are removed as part of my normal medical care may be used in medical research. ☐

I understand that my participation is entirely voluntary and that I can withdraw from the study at any time, without my medical care or legal rights being affected. If I chose to withdraw from the study, patient identifiable data or tissue collected will be excluded from the study. ☐

I give permission for my DNA to be tested for the purposes of research ☐

I agree to my surplus tissue being stored in a biobank for use in future ethically approved research. They may be used for ethically approved commercial studies, research involving animal and research outside of the UK ☐

Patient name Signature Date

Name of person taking consent Signature Date

Appendix C

Professor SM Griffin
Mr BM Dent
Northern Oesophago-Gastric Cancer Unit,
Ward 36,
Royal Victoria Infirmary,

Tel: 0191 2336161 ext. 20282

Patient Consent form

Analysis of upper gastro-intestinal cancer exudates

I have read and understood the information sheet about the above study.

I understand that my participation is entirely voluntary and that I can withdraw from the study at any time.

I allow access to my medical records by the investigators in the study and in future studies.

I have been given the opportunity to ask questions about the study.

I agree to participate in the study and that exudates and blood removed as part of my normal medical care may be used in medical research.

In the above study	☐	
In future studies	☐	that have full ethical approval

Please tick boxes to indicate your agreement

Signature	Patient name	Date
Signature	Name of consenting doctor	Date

Contact