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Molecular and Clinical Biomarkers in Gastrointestinal Cancer

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Molecular and Clinical Biomarkers in Gastrointestinal Cancer

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Doctor of Philosophy

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School of Biological Sciences

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Synopsis

Biomarkers are critically important in clinical oncology. In addition to providing valuable prognostic information, biomarkers assist in patient risk assessment, prediction of response to treatment, and monitoring progress of disease, all key factors in improving the individualisation and delivery of treatment. Furthermore, biomarkers provide insight into the mechanisms of cancer and identify novel targets for therapeutic agents. This thesis investigates both molecular biomarkers in gastroesophageal cancer and clinical biomarkers in colon cancer and identifies several molecular targets and clinical markers of interest.

Chapter 1 provides a summary of the literature on the selected biomarkers in gastrointestinal cancer, concentrating on cancer stem cells (CSC), the urokinase plasminogen activation system, and circulating tumour cells in gastroesophageal cancer, and clinical biomarkers in colon cancer.

Chapter 2 examines the role of the urokinase plasminogen activation system, including the urokinase plasminogen activator receptor (uPAR), in resected primary gastroesophageal cancers. In this comprehensive systematic review and meta-analysis, the results of 41 studies containing 2689 patients were analysed and summarised, providing level 1 evidence identifying uPA, uPAR, and plasminogen activator inhibitor 1 (PAI-1) as novel, clinically relevant, biomarkers in resected gastroesophageal cancer.

These results are then extended in **Chapter 3,** which details an immunohistochemical study exploring expression of CSC markers and uPAR on metastatic deposits of gastroesophageal cancer. CD44, a CSC marker in gastroesophageal cancers, and uPAR are shown to be independent prognostic factors associated with poorer overall survival in multivariate analysis. CD44 expression is also shown to be associated with uPAR expression providing evidence of the links between the stem cell phenotype and the uPA system.

Circulating tumour cells (CTCs) provide the essential link between the primary tumour and the distant metastatic disease. After confirming uPAR as a clinically relevant biomarker in both primary and metastatic gastroesophageal cancer, we explored uPAR as a biomarker on CTCs. Due to logistical constraints, it was necessary to develop a method for the cryopreservation of patient samples for subsequent CTC analysis. **Chapter 4** outlines a robust and feasible protocol for the delayed isolation of CTCs, and demonstrates the validity of this approach by confirming CTC enumeration on cryopreserved specimens remains an independent prognostic factor. CTC

uPAR expression did not improve on currently employed markers, but the technical issues which are the likely cause of this result are addressed.

The biology of CTCs is further explored in **Chapter 5**, in which the establishment and characterisation of two novel long term CTC cultures derived from CTCs from patients with gastroesophageal cancer are described in detail. These two cell lines exhibit distinct and contrasting phenotypic and genotypic profiles, accurately recapitulating the features of the source tumour and highlighting the marked heterogeneity seen between patients with gastroesophageal cancers. In addition to extensive characterisation, cytotoxic and radiotherapy assays on the cell lines were undertaken, with the results consistent with the molecular biology of each line, as well as the clinical picture of the source patient.

Chapter 6 examines two key clinical biomarkers in locoregional colon cancer using a large purpose built database derived from the NSW Clinical Cancer Registry, and linked to numerous government datasets to reduce bias and provide additional validity for our results. We were able to control for important confounders in our multivariate analyses including patient comorbidities and treatment received, providing a uniquely detailed analysis of population level data.

The first study explored importance of primary tumour location in early stage colon cancer. Strong evidence of an association of right sided tumours with older age and poor tumour clinicopathological factors was found. The impact of primary tumour location on overall survival varied by stage; patients with right sided colon cancer had a lower all-cause mortality in stage II, but a higher mortality in stage III disease, likely driven by underlying differences in tumour biology.

The second study examined the use of chronological age as a biomarker to select suitability for adjuvant chemotherapy in stage III colon cancer, a hotly debated and controversial topic. It was first demonstrated that elderly patients (defined as 70 years and older) were less likely to receive the standard adjuvant chemotherapy than younger patients. Multivariable cox hazard regression models were then used to show a persistent survival benefit to adjuvant oxaliplatin doublet chemotherapy in those elderly patients deemed fit enough to receive it. However this survival benefit came at the cost of a modest increase in toxicity, as reflected by an increase in hospital admissions.

Chapter 7 addresses the main findings of each study, placing the coalesced results in the context of the overall thesis and wider literature, and completes the thesis with a discussion of future directions for research.

Declaration of Authorship

I, Daniel Brungs, declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree Doctor of Philosophy, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged.

I was primarily and principally solely responsible for development of the research proposals and protocols, ethics and clinical governance submissions, selection of research methods, data analysis, interpretation of the findings, drafting and revising manuscripts, and drafting and revising the thesis.

This document has not been submitted for qualifications at any other academic institution.

This thesis is presented as a Thesis by Compilation. Chapters 2, 4, and 6 contain original manuscripts published in international peer reviewed journals. Chapter 3 and 5 contain manuscripts in preparation for publication.

Daniel Brungs

October 2018

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The work contained in this thesis was dependant on the generous contributions of many patients and their families, who gave freely in order to help others. I am inspired by their unfailing generosity and thank them for their help.

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List of Publications Produced for this Thesis

Chapter 1

1. **Brungs D**, Aghmesheh M, Vine KL, Becker TM, Carolan MG, Ranson M. Gastric cancer stem cells: evidence, potential markers, and clinical implications. Journal of Gastroenterology. 2016 Apr 1;51(4):313-26. (REVIEW) 38 Citations.

Chapter 2

2. **Brungs D**, Chen J, Aghmesheh M, Vine KL, Becker TM, Carolan MG, Ranson M. The urokinase plasminogen activation system in gastroesophageal cancer: A systematic review and meta-analysis. Oncotarget. 2017 Apr 4;8(14):23099. 6 Citations.

Chapter 3

3. **Brungs D**, Lochhead A, Iyer A, Illemann M, Colligan P, Hirst N, Splitt A, Liauw W, Vine KL, Pathmanandavel S, Carolan MG, Becker TM, Aghmesheh M, Ranson M. Expression of Cancer Stem Cell Markers is Prognostic in Metastatic Gastroesophageal Adenocarcinoma. In preparation.

Chapter 4

4. **Brungs D**, Lynch D, Luk AW, Minaei E, Ranson M, Aghmesheh M, Vine KL, Carolan M, Jaber M, de Souza P, Becker TM. Cryopreservation for delayed circulating tumor cell isolation is a valid strategy for prognostic association of circulating tumor cells in gastroesophageal cancer. World Journal of Gastroenterology. 2018 Feb 21;24(7):810. 1 Citation

Chapter 5

5. **Brungs D**, Splitt A, Aghmesheh M, Vine KL, Carolan MG, Xao J, Minai E, Becker TM, Ranson M. Establishment and characterisation of two distinct and novel long term cell cultures derived from circulating tumour cells isolated from patients with metastatic gastroesophageal cancer. In preparation

Chapter 6

6. **Brungs D**, Aghmesheh M, de Souza P, Ng W, Chua W, Carolan M, Clingan P, Healey E, Rose J, Tubaro T, Ranson M. Sidedness is prognostic in locoregional colon cancer: an analysis of 9509 Australian patients. BMC Cancer. 2017 Dec;17(1):251.16 Citations.
7. **Brungs D,** Aghmesheh M, de Souza P, Carolan M, Clingan P, Rose J, Ranson M. Safety and Efficacy of Oxaliplatin Doublet Adjuvant Chemotherapy in Elderly Patients With Stage III Colon Cancer. Clinical Colorectal Cancer.2018, 17 (3), e549-e555.

List of Frequently Used Abbreviations

CK	Cytokeratin
CRC	Colorectal cancer
CSC	Cancer stem cell
CTC	Circulating tumour cell
DAPI	4′,6-diamidino-2-phenylindole
EpCAM	Epithelial cell adhesion molecule
EMT	Epithelial-mesenchymal transition
GI	Gastrointestinal
GOC	Gastroesophageal cancer
GIST	Gastrointestinal stroma tumour
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
LsCC	Left sided colon cancer
MMP	matrix metalloproteases
OS	Overall Survival
PAI	Plasminogen activator inhibitor
PBMC	Peripheral blood
RsCC	Right sided colon cancer
SCC	Squamous cell carcinoma
SEER	Surveillance, Epidemiology, and End Results
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor

Table of Contents

Synopsis **i**

Declaration of authorship **iii**

Acknowledgements **iv**

List of publications included as part of the thesis **v**

Abbreviations **vi**

Table of contents **vii**

Chapter 1. Background and aims **1**

1.1 Gastrointestinal cancers 2

1.2 Biomarkers in gastroesophageal adenocarcinoma 5

1.3 Clinical biomarkers in colon cancer 32

1.4 Aims and objectives 34

Chapter 2. Expression of the uPA system in resectable gastroesophageal cancer **58**

2.1 The urokinase plasminogen activation system in gastroesophageal cancer: A systematic review and meta-analysis. 59

Chapter 3: Expression of cancer stem cell markers and uPAR in metastatic gastroesophageal cancer **87**

3.1 Expression of Cancer Stem Cell Markers is prognostic in Metastatic Gastroesophageal Adenocarcinoma. 88

3.2 Appendix: uPAR expression on metastatic GOC 101

Chapter 4: Circulating Tumour Cell Enumeration in gastroesophageal cancer **115**

4.1 Cryopreservation for delayed circulating tumor cell isolation is a valid strategy for prognostic association of circulating tumor cells in gastroesophageal cancer 116

4.2 Appendix: Expression of uPAR on CTCs in gastroesophageal cancer 130

Chapter 5: Circulating Tumour Cell Culture in gastroesophageal cancer **138**

5.1 Establishment and characterisation of two distinct and novel long term cell cultures derived from circulating tumour cells isolated from patients with metastatic gastroesophageal cancer. 139

Chapter 6: Clinical Biomarkers in logoregional colon cancer **164**

6.1 Sidedness is prognostic in locoregional colon cancer: An analysis of 9509 Australian patients 166

6.2 Safety and Efficacy of Oxaliplatin Doublet Adjuvant chemotherapy in Elderly Patients with Stage III Colon Cancer 181

Chapter 7: Discussion **198**

7.1 Summary of principle findings 199

7.2 Conclusions 206

Appendices

1. Gastric cancer stem cells: evidence, potential markers, and clinical implications. *Journal of Gastroenterology*.
2. The urokinase plasminogen activation system in gastroesophageal cancer: A systematic review and meta-analysis. *Oncotarget*.
3. Cryopreservation for delayed circulating tumor cell isolation is a valid strategy for prognostic association of circulating tumor cells in gastroesophageal cancer. *World Journal of Gastroenterology.*
4. Sidedness is prognostic in locoregional colon cancer: an analysis of 9509 Australian patients. *BMC Cancer*
5. Safety and Efficacy of Oxaliplatin Doublet Adjuvant Chemotherapy in Elderly Patients With Stage III *Clinical Colorectal Cancer*

Chapter 1

Background and Aims

Publication Details: Brungs D, Aghmesheh M, Vine KL, Becker TM, Carolan MG, Ranson M. Gastric cancer stem cells: evidence, potential markers, and clinical implications. Journal of Gastroenterology. 2016 Apr 1;51(4):313-26. (REVIEW)

Contribution of authors:

DB –concept development, data collection and analysis, interpretation of results, manuscript draft and revisions

TB, MC, KV, MA – interpretation of results, manuscript revisions

MR – concept development, interpretation of results, manuscript draft and revisions

1.1 Gastrointestinal cancers

Gastrointestinal cancers are a leading cause of cancer related death. They arise in the gastrointestinal tract and accessory digestive organs of digestion, including the oesophagus, stomach, biliary system, pancreas, small intestine, large intestine, rectum and anus. Gastrointestinal cancers are classified and treated according to anatomical site of origin. This thesis focuses on gastric, oesophageal and colon cancer.

1.1.1 Gastric and oesophageal cancer

Gastroesophageal cancers are the most common gastrointestinal malignancy worldwide. Gastric adenocarcinoma is the fifth most common type of cancer and the third leading cause of cancer-related death worldwide, and oesophageal cancer is the eighth most common cancer worldwide [1,2]. Gastric and oesophageal cancers differ in incidence, geographic distribution, and aetiology. Most gastric cancers are adenocarcinomas, although are highly heterogeneous in molecular pathogenesis. Gastrointestinal stromal tumours (GIST), lymphomas, and neuroendocrine tumours are found in a small minority of cases [3]. There are several key risk factors for gastric cancer. A small proportion of gastric cancers are due to heritable causes such as E-cadherin gene (CDH1) mutations (leading to hereditary diffuse gastric cancer) and DNA repair enzyme deficiency in Lynch Syndrome [4]. The primary risk factor for most sporadic distal gastric cancers is chronic inflammation caused by *Helicobacter pylori* infection [5-7].
The histology of oesophageal cancer varies by location; most upper and middle third oesophageal cancers are squamous cell carcinomas (SCC), while the majority of lower oesophageal and gastroesophageal junction (GEJ) tumours are adenocarincomas. Cigarette smoking, alcohol consumption, and diets low in vegetables and fruits are the predominate causes of oesophageal SCC [8]. The aetiology and incidence of gastroesophageal cancers in Western countries is changing, with upper oesophageal SCC and distal gastric cancer becoming more uncommon, while incidence of adenocarcinomas of gastric cardia and gastroesophageal junction are rapidly rising [9,10]. This is thought to be due to lifestyle changes with increasing obesity and gastroesophageal reflux disease [8].
The Cancer Genome Atlas has described 4 major gastroesophageal adenocarcinoma subtypes (Figure 1). Of note, oesophageal SCC (ESCC) is a distinct disease entity and is not discussed further in this thesis. 1) EBV tumours which are positive for the Epstein Barr virus (EBV). These tumours display recurrent PIK3CA mutations, extreme DNA hypermethylation, JAK2 amplification, and overexpression of PD-L1 and PD-L2. 2) Microsatellite unstable tumours (MSI) with high mutation rates including in key targetable oncogenic pathways. 3) Genomically stable (GS) tumours with mutations of RHOA or fusions involving RHO-family GTPase-activating proteins. 4) Tumours with chromosomal instability (CIN), with TP53 mutation, RTK-

RAS activation and marked aneuploidy [11]. Analysis of oesophageal adenocarcinomas show marked similarity with the CIN subtype of the gastric cancers, suggesting these tumours can be considered a single disease entity [12].

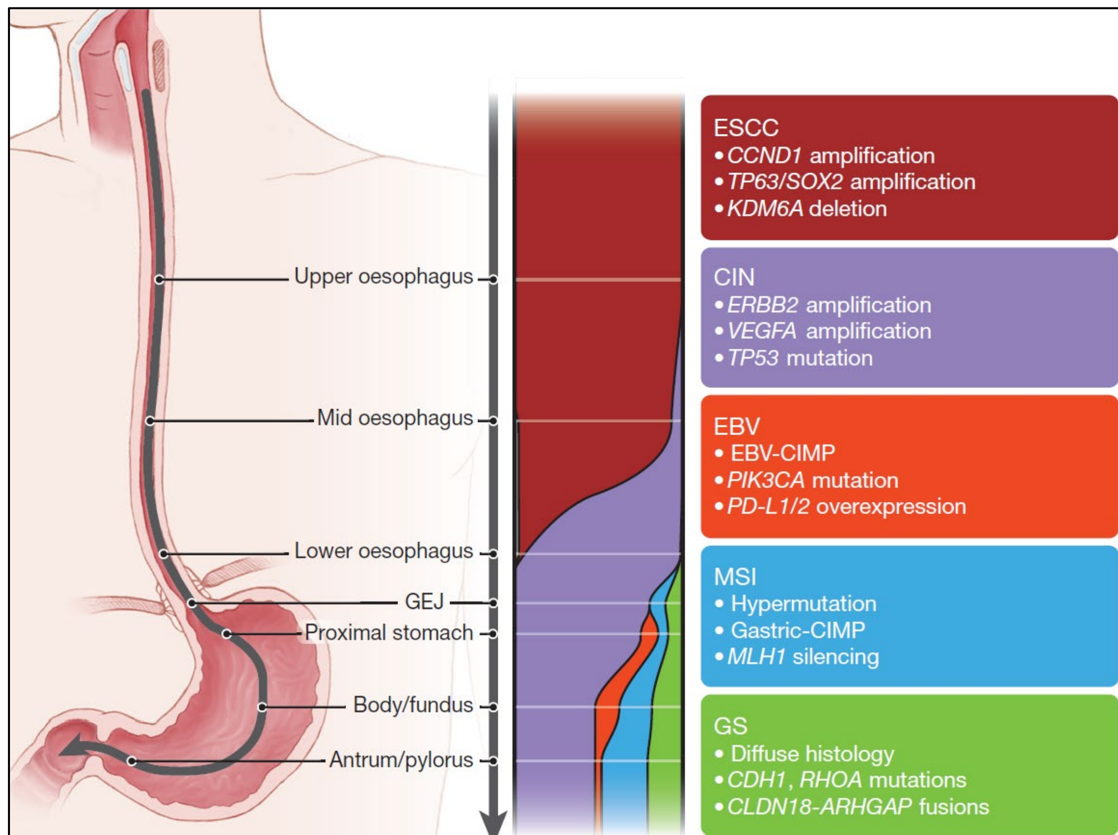

Figure 1: Gradation of molecular subclass of gastroesophageal carcinoma.
Schematic representing shifting proportion of subtypes of gastroesophageal carcinoma from the proximal oesophagus to the distal stomach. The widths of the colour bands represent the proportion of the subtypes present within anatomic regions. Taken from The Cancer Genome Atlas [12] .

Similarly, despite differences in epidemiology, currently employed systemic treatments have not shown significant differences in efficacy or toxicity between distal oesophageal, gastroesophageal and gastric adenocarcinoma. Consequently these malignancies are treated in a similar fashion in the advanced setting, with most clinical trials including patients with distal oesophageal, GEJ, and gastric adenocarcinomas [13].

1.1.2 Colorectal cancer

Colorectal cancer (CRC) is one of the most common cancers worldwide and projected to account for 13% of all new cancer cases diagnosed in Australia in 2015[14]. While CRC remains the fourth most common cause of cancer death worldwide, mortality is improving in Western countries due to the institution of cancer screening programs with the subsequent detection and removal of adenomas and early cancerous lesions[15].

CRC is a disease related to aging, with almost 40% of CRC diagnosed above the age of 75 years in Australia, and occurring only rarely in patients younger than 40[16]. Apart from age, there are two broad categories of risk factors for developing colon cancer, genetic determinants and lifestyle factors. A minority of colon cancers (5-10%) are due to inherited syndromes such as Familial Adenomatous Polyposis (FAP) or Lynch associated syndromes[17]. In addition, patients with a personal history of adenoma, colon cancer or inflammatory bowel disease, as well as a significant family history of CRC, are considered at high risk of colon cancer and recommended to undergo screening[18]. There are a large number of lifestyle factors which are associated with a small increased risk in CRC including obesity, diabetes mellitus, red and processed meat, smoking and alcohol intake[18].

The molecular pathogenesis of CRC is a well characterised multistep process of inherited or acquired genetic mutations driving the progression of normal colon epithelium to invasive cancer via the intermediate precursor lesion, the adenomatous polyp[19]. Three key pathways have been identified in CRC tumorigenesis. The chromosomal instability pathway (CIN) is characterised by gross chromosomal abnormalities, from reduced activity of tumour suppressor genes or activation of growth promoting pathways, due to inherited (typified by FAP) or sporadic mutations[20,21]. In the DNA mismatch repair (MMR) pathway, seen in both Lynch syndrome and sporadic CRCs, cells with aberrant DNA repair pathways, due to germline mutations or epigenetic hypermethylation of the MMR gene promotor, accumulate widespread DNA errors throughout the genome, presenting phenotypically with high levels of microsatellite instability[22]. The hypermethylation phenotype (CIMP positive) is a distinct subtype of CRC, characterised by CpG island hypermethylation and serrated adenoma precursors, associated with microsatellite instability, BRAF mutations, and poor clinical outcomes [23].

While there are a number of important prognostic factors identified in CRC, tumour stage at diagnosis remains the most important prognostic variable. While stage I colon cancer is cured with surgical resection alone, stage II and III colon cancer is usually treated with curative intent using a combination of surgery and adjuvant chemotherapy [18]. Although Stage IV disease was

traditionally treated as palliative, aggressive local treatment of metastatic disease, combined with chemotherapy and biological treatments (including VEGF and EGFR targeted agents) have markedly improved patient outcomes [24].

1.2 Biomarkers in gastroesophageal adenocarcinoma

Tumour, node and metastasis (TNM) stage is the most important prognostic factor in gastroesophageal cancers [25]. Multimodality approaches which include chemotherapy, radiotherapy, and surgical resection offer potential cure in localised disease [26]. However the majority of patients with gastroesophageal cancer present with locally advanced or metastatic disease [27]. Despite improvements in systemic treatments, and numerous active systemic agents, prognosis remains poor with median overall survival of less than 12 months [28].

There is a paucity of biomarkers in gastroesophageal cancer to guide systemic treatment. Molecular classification using the HER2 status is the sole routine tissue biomarker currently used in gastroesophageal cancers. HER2 amplification or overexpression is seen in 22% of patients with advanced disease[29]. There is contrasting evidence regarding the prognostic importance of HER2 status, with some but not all studies reporting worse prognosis [30-32]. The TOGA trial demonstrated improved overall survival and progression free survival with the addition of trastuzumab, a monoclonal antibody against HER2, in combination with chemotherapy in HER2 positive locally advanced or metastatic gastroesophageal cancers, leading to routine incorporation of HER2 testing in all advanced gastroesophageal cancers [33]. There is an increasing focus on the use of gene signatures as biomarkers to predict response to chemotherapy[34]. While retrospective data is encouraging, prospective studies to validate these findings are required prior to clinical use.

Similarly, there are very limited circulating biomarkers currently in clinical use. While monitoring of blood levels of carcinoembryonic antigen (CEA) and Cancer Antigen 19.9 (Ca19.9) is routinely preformed in clinical practice to monitor disease response, these serum markers are limited by poor sensitivity and specificity, and may not always reliably reflect responses to treatment [35].

There are several key biomarkers identified from the literature that show promise as potential clinically relevant biomarkers in gastroesophageal cancer. These include cancer stems cells (CSC), the plasminogen activation system (PAS) and circulating tumour cells (CTCs) and are discussed below.

1.2.1 Cancer Stem cells in Gastroesophageal cancer

1.2.1.1 Cancer Stem Cells (CSC)

A key oncological issue is whether cancer growth is driven by the majority of tumour cells, or by a rare subpopulation of CSCs. There are several proposed models. The clonal evolution model states that each cell within a tumour has equal potential to acquire genetic/epigenetic changes which confer growth advantages and subsequent new tumour growth[36]. The CSC model on the other hand proposes that the growth of a tumour is driven by a small population of self-sustaining cells with the stem-cell properties of longevity, infinite proliferation, and ability to differentiate into the entire heterogeneous population of the tumour [37]. Integral to the CSC model is a subpopulation at the apex of the hierarchy (usually comprising less than 5% of the cancer) responsible for the formation, maintenance and continued growth of the tumour [38]. Stem cells can symmetrically divide into self- renewing identical daughter stem cells with self-renewal capacity, or asymmetrically divide to both a differentiated progenitor cell and a stem cell [39] .

Recent refinements to the CSC theory propose a more dynamic model, with a fluid CSC population regulated by the tumour-cell environment, rather than a rigid hierarchical structure [37,40]. It is now apparent that a CSC phenotype can be induced in differentiated cancer cells by exposure to growth factors secreted from stromal cells, suggesting a bidirectional pathway between the CSC and differentiated cell populations [41,42]. Furthermore, some types of leukaemic stem cells have been shown to be subjected to clonal evolution [43]. The reacquisition of self-renewal properties in non-CSC populations, in addition to genotypic and phenotypic heterogeneity within CSCs, highlight that the CSC population is fluid in both numbers and character (Figure 2).

Cancer stem cells are defined functionally rather than from cellular origin, with CSCs having superior tumour initiating, growth, and metastatic potential than other tumour cells [44]. *In vitro* studies with cultured gastric CSCs indicate these cells to be more resistant to chemotherapy and radiotherapy [45,46], possibly due to high expression of anti-apoptotic proteins, increased efficiency of DNA repair, and alterations in cell cycle kinetics [37,39] . CSCs are responsible for the renewal of tumour mass following systemic treatment, and the development of treatment resistant subclones [39]. The CSC's long proliferative lifespan and repeated DNA replication events renders them more susceptible to further mutations and epigenetic changes creating further malignant clones [47].

The first definitive evidence of a CSC population was provided by Bonnet *et al* (1997), who

demonstrated that a cell population defined by the CD34+/38- phenotype was able to serially reproduce acute myeloid leukaemia in immunodeficient mice [48]. Crucially this cell population did not only self-renew, but could reconstitute the full spectrum of cell populations seen in AML. This approach has been used to isolate CSCs in solid malignancies including breast cancer [49], prostate cancer [50], pancreatic cancer [51,52], melanoma [53], colon cancer [54,55], brain cancer [56,57], and liver cancer [58], supporting the model of cancer growth initiated and maintained by CSCs. The presence of CSCs in solid malignancy has been confirmed with lineage tracing studies which identified a subpopulation of cells which reconstitute the entire tumour following chemotherapy [59].

Experimental evidence for CSCs must demonstrate both self-renewal and ability to differentiate into the heterogeneous cell population that constitute a tumour [60]. Serial transplantation in animal models fulfils these criteria and is proposed as the best functional assay to identify CSCs [60]. An alternative experimental model is *in vitro* spheroid colony formation of candidate CSCs in culture media, as continued growth of colonies indicates self-renewal [61]. Although serial passage in animal models is considered the gold standard, the two methods seem to provide similar results when identifying candidate CSCs [61].

The CSC model has important clinical implications, as it infers that anti-neoplastic treatments should focus on eliminating both a small population of CSCs within the tumour, as well as the rapidly dividing but terminally differentiated bulk of cancer [60]. Figure 2 illustrates the various models schematically.

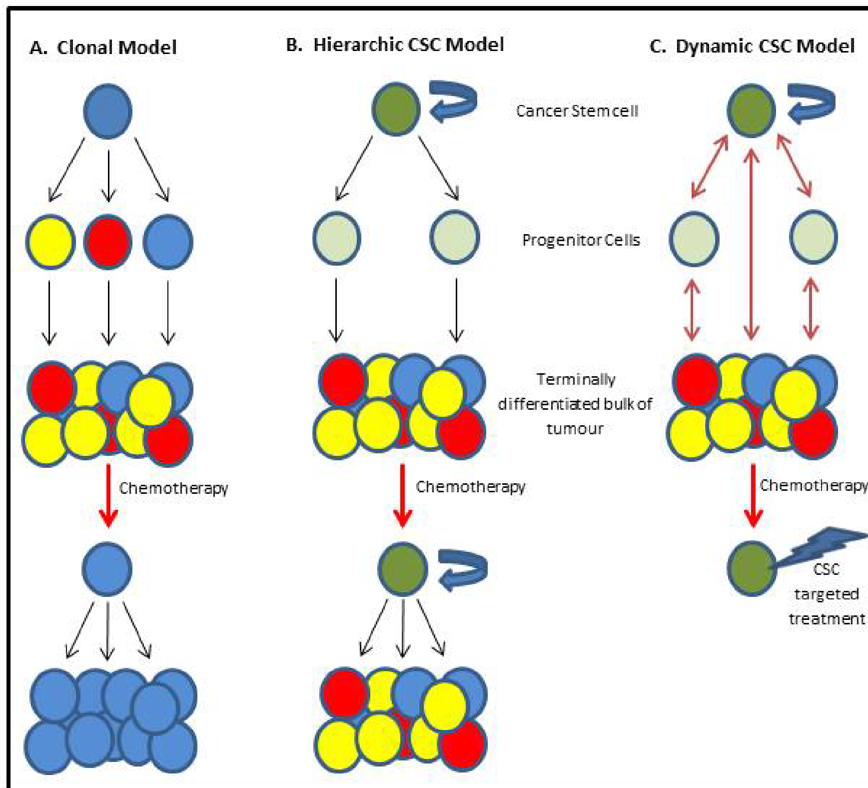

Figure 2: Three models of tumour growth and their clinical implications (a) The clonal model, where each cell has the potential to acquire additional mutations which confer a growth advantage. Chemotherapy selects a treatment resistant subclone which subsequently reconstitutes the tumour mass. (b) The hierarchic cancer stem cell (CSC) model, whereby a small population of self-renewing CSCs are responsible for all tumour growth, giving rise to progenitor cells, which subsequently de-differentiate into the bulk of the tumour which has lost capacity to self renewal. The chemo-resistant CSC population is enriched by chemotherapy, and is able to restore the tumour bulk. (c) The dynamic CSC model is a more refined CSC model, demonstrating the bi-directional flow of cells between the stem cell and differentiated compartments. This model highlights the need to combine a CSC targeted agent with chemotherapy. The CSC targeted agent eliminates the chemo-resistant CSC population preventing the reconstitution of the tumour bulk, while the chemotherapy reduces the tumour bulk of differentiated cells, stopping these cells de-differentiating to replenish the CSC population.

1.2.1.2 CSC and metastases

The CSCs unique properties of self-renewal and multi lineage differentiation suggest a likely role in the initiation and progression of distant metastatic disease. Although there is no direct experimental evidence of CSCs as the origin of metastases many studies provide supporting data [62]. The presence of unique tumour subpopulations with CSC markers have been shown to be integral for the development of metastatic disease in a variety of malignancies including pancreatic [51], colorectal [63], and breast [64] cancer, as depletion of this population prevented the metastatic spread of the tumour [51]. Dieter *et al* demonstrated differential contributions of individual CSC clones to the growth of primary and metastatic tumours, and identified a subpopulation of CSCs in colon cancer solely responsible for metastases formation [65]. In addition, immunohistochemical studies in gastric cancer have shown an increased risk of metastatic disease associated with CSC marker expression in the primary tumour [66,67].
A proposed mechanism underlying the progression of cancer to metastases is the epithelial-mesenchymal transition (EMT) [68]. EMT is the process whereby tumour cells lose epithelial characteristics and acquire a mesenchymal phenotype to facilitate cancer metastasis and survival. It becomes more and more evident that EMT and CSC phenotypes are largely overlapping and provide properties of invasion, tumour seeding, drug resistance, and survival. It is thought that CSCs in primary tumours can metastasise to distant sites via EMT [69]. Furthermore a CSC phenotype can be obtained by inducing an EMT state [70,71]. Tumour cells disseminated in the blood (circulating tumour cells) are enriched for both an EMT and CSC phenotype [72]. Gastric cancer patients who have detectable circulating tumour cells (CTCs) which express CSC markers have a poorer prognosis than those with CTCs without CSC markers [73]. This is reinforced by clinical evidence of an association between EMT and CSCs, with immunohistochemical expression of CD44, a gastric CSC marker, significantly correlated with expression of EMT markers such as Snail-1 and ZEB1 in resected gastric cancer [74]. Gastric CSCs isolated from a cell line showed increased expression of EMT markers (including Snail, Twist, and vimentin) and CD44 [75]. Moreover, analysis of combined expression of CD44 with EMT markers was predictive of a poorer disease free survival and overall survival (OS) in a multivariate model, consistent with the aggressive phenotype of cells expressing CSC and EMT markers [74].

1.2.1.3 Identification of Cancer Stem Cells

A key issue in the study of CSCs is development of reproducible and reliable methods for CSC isolation and identification. The American Association for Cancer Research (AACR) defines CSCs as subpopulations of cells within a tumour that possess the capacity for self-renewal and cause the heterogeneous lineage of cancer cells that constitute the tumour [60]. As discussed above, the experimental methods used to confirm a population of cells as CSCs are serial

passage of tumours in animal models, or tumour-spheroid assays. Animal model serial transplantation is regarded as the gold standard as it is considered a physiologically relevant functional assay to demonstrate self-renewal and lineage capacity [60]. However some groups have questioned this paradigm as the rarity of CSCs found in human cancers may be due to an inadequate local environment in the xenograft [76,77]. Tumour spheroid assays are a more rapid method (as serial transplantation can take several months), and have been shown to increase expression of stem cell markers, but have potential pitfalls as not all isolated CSCs form spheroids [46,78].

There are many challenges in identifying a CSC population within a tumour. Firstly, as discussed above, the CSC population is dynamic with bidirectional flow between the CSC and differentiated cell populations. Secondly, the currently utilised CSC markers are not specific, and are expressed on non-malignant cells, as well as early progenitor cells which have lost stem cell features but retained phenotypic markers. Thirdly, multiple populations of CSCs may exist within a tumour mass, and combinations of multiple markers may be required to identify the complete CSC population.

Candidate CSCs are currently identified predominantly by two methods: the side population assay or expression of CSC surface markers.

1.2.1.3.1 Side Population Assay

The side population (SP) assay identifies the fraction of cells that efflux Hoechst dye by ATP binding cassette (ABC). It is a highly preserved marker of stem cells across a variety of tissues and tumours, with a higher capacity for self-renewal, leading some authors to suggest that the SP subset may represent a universal CSC population [79]. However the SP assay is hampered by poor specificity, with differentiated adults cells in the gastrointestinal tract demonstrating a SP phenotype [80].

There are inconsistent results regarding SP assays as a potential CSC marker in gastric cancer. Zhange *et al* showed CSC properties in SP cells from the MKN-45 cell line, but not from the BGC-823 cell line [81] . Although some studies in gastric cancer lines have shown CSC properties in the SP [82,83], others have found no difference compared to a non-SP subset [45,84,85]. Overall the utility of the SP assay to identify gastric CSC remains controversial.

1.2.1.3.2 Expression of Cell Surface Markers

An integral tool in the identification and isolation of candidate CSCs is the expression of unique combinations of cell surface markers. This approach has allowed isolation of CSCs in many solid malignancies by flow cytometry or magnetic cell sorting. Currently identified CSC

markers are expressed in an overlapping manner on a variety of tumours, as well as normal stem cells. A summary of the most common markers described for solid tumours is provided in Table 1. Many of these potential markers are found in gastric cancer.

Table 1: Cell Surface Expression of Cancer Stem Cell Markers in Different Solid Tumour types.

Tumour	Described Cell Markers	Reference
Colon	CD133, CD44, CD166. EpCam, CD24, ALDH1	Botchkina 2013 [86]
Pancreas	CD133, CD44/CD24, ALHD1	Zhan *et al* 2015 [87]
Breast	CD44+/CD24-, ALDH1	Carrasco *et al* 2014 [88]
Brain	CD133, CD44	Jackson *et al* 2015 [89]
Lung	CD133	Lundin *et al* 2013 [90]
Melanoma	CD20, CD133, CD271	Lang *et al* 2013 [91]
Prostate	CD44+/CD24-, CD133, ALDH,	Sharpe *et al* 2013 [92]

1.2.1.4 Gastric Cancer Stem Cells

1.2.1.4.1 Origin of gastric cancer stem cells

The origin of gastric CSCs remains uncertain. A possible source of gastric CSCs is gastric stem cells which have lost regulated quiescence. The existence of multipotent gastric stem cells which give rise to all major epithelial cell types has been demonstrated in mouse models [93]. There are multiple populations of gastric stem cells. The Lgr5+ cells arise at the base of the gastric gland and continuously differentiate into all antral unit cells, while the Villin+ cells are located at the isthmus and are a quiescent stem cell population which require cytokine stimulation to activate, acting as a stem cell source if the Lgr5+ cells are damaged [94-96]. Sox2+ cells, present in the antrum and corpus, are able to differentiate into all cell types found in a gastric unit, and ablation of the Sox2+ population prevents renewal of gastric epithelium [97]. Further populations of differentiated gastric cells, such as chief cells expressing the marker Troy, have been shown to de-differentiate and function as multipotent stem cells, acting as reserve stem cell populations [98].

Aberrant genetic and epigenetic mutations in these gastric stem cells, in conjunction with stimulating factors from the microenvironment, may lead to the formation of CSCs [47]. For example, APC deletion in Lgr5+ stem cells led to rapid development of adenomas in a mouse model due to expansion of the stem cell compartment [95]. Wu *et al* demonstrated co-localisation of CSC markers (CD26, CD44, ALDH1, CD133) with Lgr5+ cells in gastric cancer suggesting

they may be functionally related [94]. Similarly Sox2 expression is altered during gastric cancer pathogenesis [99,100], although reports are contradictory as to whether Sox2 is overexpressed and oncogenic [101,102], or downregulated and anti-oncogenic, with lower Sox2 levels associated with poorer clinical outcomes [99,103]. Another proposed gastrointestinal stem cell marker, doublecortin-like kinase (Dclk1), is highly expressed on cells in the stem cell zone of mouse gastric glands [104]. K-ras induced chronic inflammation in K19-K-ras-V12 transgenic mice led to expansion of the Dclk1+ cell population during the development of high grade dysplasia [105].

An alternative hypothesis suggests that gastric CSCs arise from bone marrow derived mesenchymal stem cells, pluripotent adult stem cells which are recruited to peripheral organs in response to chronic inflammation. Their function is to assist in regeneration after failure of local stem cells [106]. Bone marrow derived cells (BMDC) have been shown to repopulate gastric mucosa in response to chronic inflammation due to *H. Pylori* infection, and may contribute to carcinogenesis [107,108]. In a mouse model infected with *H. pylori*, almost a quarter of high grade dysplastic gastric lesions included BMDC [109]. The BMDC are proposed to differentiate in the gastric mucosa by cell-cell fusion with local gastric epithelial cells, and in the context of further chronic inflammation, induce EMT and the emergence of CSCs [106,110]. It is important to note however, despite these provocative findings, the majority of dysplastic lesions do not arise from BMDC. Further studies are required to fully explore the pathogenesis of gastric CSCs.

1.2.1.4.2 Gastroesophgeal CSC markers:

1.2.1.4.2.1 CD44 and CD44 variants

CD44 is a transmembrane glycoprotein expressed on leukocytes, endothelial cells, hepatocytes, and mesenchymal cells, and has a variety of physiological roles including matrix adhesion, cell migration, and differentiation [111]. CD44 is encoded by the 20 exon *CD44* gene, with exon 1-5 encoding the constant region of the extracellular domain, and exon 16-20 spliced together to form the standard isoform [112]. The variant exons 6-15 are subject to alternative splicing and can be assembled in different combinations with the standard exon to make variant isoforms [112]. CD44 variants (designated as CD44v) have been proposed as a more specific CSC marker than CD44 due to their more restricted distribution pattern. Generally, expression of CD44v on gastric cancers cells is well correlated with CD44 expression [66,112].

CD44 has been proposed to mediate signal transduction of oncogenic pathways such as the human epidermal growth factor receptor (HER) pathway [113]. Cancer cells with high CD44 expression have an enhanced resistance to reactive oxygen species due to increased glutathione synthesis and upregulation of anti-oxidant genes [114]. The first evidence of gastric CSCs was demonstrated with the self-renewal and heterogeneous linage of a CD44+ subpopulation [45].

There are now numerous studies which support CD44 as a marker for CSC in gastric cancer (Table 2). Although most studies support CD44 as a CSC marker, two studies were unable to demonstrate stem-cell properties in the CD44+ purified subset of patient derived gastric cancer cells, perhaps due to inappropriate microenvironment in mouse models [115,116]. CSC populations have also been identified using a combination of CD44+ and other markers including EpCam [112,117], CD54+ [118], and CD24 [119].

Despite this, CD44 is not a specific or sensitive marker for gastric CSC. The true CSC population has been estimated at <5% of CD44+ cells [45], and CD44 is widely expressed on non-malignant tissue. Other markers are required in addition to CD44 to improve the specificity of CSC identification.

There is only limited evidence of CD44v as a CSC marker. Lau *et al* showed CD44v8-10 was the predominate CD44v expressed on CD44+ gastric cancer cells (79% of CD44+ cells), and demonstrated self-renewal and heterogeneous linage in serial transplants of CD44v8-10 in mouse models [112]. Although the CD44v8-10+ fraction was more tumourigenic in mouse models, both the CD44v8-10+ and CD44v8-10- cells were able to form tumour spheres [112]. While CD44v appears to be a more specific marker for gastric cancer than CD44, more research needs to be performed to elucidate their biological role and confirm CSC characteristics.

Table 2: Summary of cancer stem cell markers in gastric cancer (*in vitro and mouse model studies).*

Stem Cell Characteristic	Marker	References
Self –Renewal (serial transplantation in mouse models, or maintained in serial? spheroid culture for weeks)	CD44+	Takaishi *et al* 2009 [45],
	CD44v8-10	Lau *et al* 2014 [112]
	CD133+	Nil study identified
	ALDH1	Katsuno *et al* 2012 [120], Nishikawa *et al* 2013 [121]
Multi lineage differentiation	CD44+	Takaishi *et al* 2009 [45],
	CD44v8-10	Lau *et al* 2014 [112]
	CD133+	Fukamachi *et al* 2011 [122]
	ALDH1	Katsuno *et al* 2012 [120]
Increased resistance to chemotherapy/radiotherapy	CD44+	Takaishi *et al* 2009 [45], Zhang 2011 [119], Yoon *et al* 2014 [123],
	CD133+	Zhu *et al* 2014 [124]
	ALDH1	Zhi *et al* 2011 [125], Nishikawa *et al* 2013 [121]
Increased tumourigenicity (faster growing tumours, or smaller tumour seeding volume)	CD44+	Takaishi *et al* 2009 [45], Song *et al* 2011 [119],
	CD133+	Nil study identified
	ALDH1	Zhi *et al* 2011 [125], Katsuno *et al* 2012 [120], Nishikawa *et al* 2013 [121],
Attenuation of stem cell characteristics with knock-down model	CD44+	Takaishi *et al* 2009 [45]
	CD133+	Zhu *et al* 2014 [124]
	ALDH1	Nil Study identified
Upregulated stem cell or mesenchymal markers	CD44+	Yu *et al* 2014 [126]
	CD133+	Song *et al* 2011 [127]
	ALDH1	Zhi *et al* 2011 [125], Nishikawa *et al* 2013 [121],

1.2.1.4.2.2 CD133

CD133 is a transmembrane glycoprotein plasma membrane protein found on embryonic epithelial structures and hematopoietic stem cells [128]. It is proposed to function as an organiser of plasma membrane topology, and have a role maintaining appropriate lipid composition of plasma membrane [129]. CD133 has been identified as a CSC marker in a variety of solid tumours (Table 1).

Most studies have identified CSCs using AC133, an antibody which detects a glycosylated epitope of CD133 [130]. Some authors have recommended caution using CD133 as a marker to identify CSC after they showed downregulation of CD133 epitopes (including the target of

AC133) during differentiation. However constant CD133 protein expression suggests differentiated cells may express CD133, but with masked epitopes due to differential glycosylation [131]. Post translational modification of CD133 may have roles in invasion and metastasis, and influence antibody binding by altering the epitope's accessibility [130].

Consequently there is debate regarding the utility of CD133 as CSC marker in gastric cancer. Although some studies have demonstrated CSC properties with the CD133+ subpopulation, several groups have found contrasting results, with CD133- cells able to initiate tumours [45,112,115] (Table 2).

1.2.1.4.2.3 ALDH1

Aldehyde Dehydrogenase (ALDH) is a family of enzymes that have a role in cellular detoxification, differentiation and drug resistance via oxidation of cellular aldehydes [132]. ALDH1 functions as a modulator of cell proliferation and stem cell differentiation, and is a marker of CSCs in a variety of cancers (Table 1). High activity of ALDH1 confers resistance to chemotherapeutic agents [133]. ALDH^{high} cell populations are identified with the Aldeflour assay or by ALDH1 antibody, and have been shown to correlate with CD44 expression [121].

Katsuno *et al* [120] demonstrated CSC properties of ALDH1+ cells isolated from gastric cancer cell lines including self-renewal, heterogeneous linage and increased tumourogenicity. Interestingly, in contrast to other cancers, they found TGF-β inhibited the function of the CSC population[120]. Studies demonstrating the CSC properties of ALDH1 in gastric cancer are summarised in Table 2.

1.2.1.4.2.4 Other potential CSC markers

Numerous other molecules have been identified as potential gastric CSC markers and these are addressed below. However, it must be borne in mind that the evidence for these molecules as markers is either limited or contradictory. Further studies are thus required to either confirm or refute their utility as markers of the CSC population.

CD24 is a cell surface adhesion molecule expressed on leukocytes, normal gastric parietal cells, and intestinal stem cells [134]. CD24 expression is associated with aggressive clinicopathological features in gastric cancer, and facilitates cell migration and invasion of gastric cancer cells [134,135]. Evidence for CD24 as a CSC marker in gastric cancer is conflicting. Zang *et al* found that the CD44+/CD24+ fraction isolated a CSC population in gastric cancer cell lines [119], while Takaishi *et al* was unable to find evidence of CSC characteristics in a CD24+ population in spheroid and mouse models [45].

The epithelial cellular adhesion molecule (EpCam) is a transmembrane glycoprotein detected in the majority of epithelial tissues, with roles in cell adhesion, signalling, migration, proliferation and differentiation [136]. EpCam is overexpressed in gastric cancer and gastric cancer cell lines [121,137]. Several studies have shown that gastric CSCs lie within the EpCam+ population, with the EpCam- population unable to form tumours in mouse models or tumourspheres [112,117]. However, additional more specific markers are required in addition to EpCam as the majority of gastric cancer cells are EpCam positive.

CD49f is a subunit of laminin receptors which has been used to isolate CSCs in prostate, breast, brain and colon cancers [116]. Fukamachi *et al* demonstrated CSC properties of self-renewal, heterogeneous linage, and chemotherapy resistance to the CD49f selected cells from primary gastric cancer mouse xenografts [116]. Further studies are needed to confirm CD49f stem cell properties.

CD54 (also known as intercellular adhesion molecule-1; ICAM-1) is an adhesion molecule essential for arrest and transmigration of leukocytes out of blood vessels, and is widely expressed on immune, stromal and malignant cells[138]. Decreased CD54 expression on resected gastric cancer is associated with poorer prognosis and increased risk of lymphatic spread [139]. CD44+/54+ cells that were isolated from primary gastric cancers and peripheral blood samples, demonstrated superior tumourigenicity, multiple linage capability and self-renewal, compared to CD44- or CD54- cells, suggestive of a CSC population in both the primary tumour and in the circulation [118].

CD90 is expressed in bone marrow derived mesenchymal stem cells, haematopoietic stem cells, keratinocytic stem cells, and has been used to identify CSC populations in liver, breast and brain cancer [140]. CD90+ cells isolated from patient derived gastric cancer xenografts demonstrated self-renewal and a heterogeneous linage [140]. CSCs obtained by preconditioning a gastric cancer cell line with chemotherapy displayed increased expression of CD90 as well as bonafide CSC markers [75].

CD71 (also known as the transferrin receptor) is a membrane protein highly expressed on myocytes, keratinocytes, hepatocytes, pancreatic cells, and erythroid precursors, with a physiological role in mediating the uptake of transferrin-iron complexes. CD71 has been proposed as a negative selection marker, with the CD71 negative subpopulation of a gastric cancer cell line displaying chemoresistance, self-renewal, heterogeneous linage, and increased tumourigenicity in mouse models [141].

Finally, several transcription factors, including Sox2, Oct4, and Nanog are expressed on gastric stem cells, and have been proposed as potential CSC markers. Gastric CSCs enriched by the side population assay or spheroid formation have a higher expression of Sox2, Oct4 and Nanog compared with parental cells [46]. As discussed above however, studies evaluating the association between clinicopathological variables with immunohistochemical expression of Sox2 in resected gastric cancer have shown conflicting results [99,100,103,142]. Similarly, although some studies have shown poorer prognosis with Oct4 expression in resected gastric cancer [143,144], another large patient series found the opposite result [101].

1.2.1.5 Clinical Implications of Gastric Cancer Stem Cells

1.2.1.5.1 Gastric CSC marker expression and patient prognosis

CSC marker expression in cancer tissue is emerging as a clinically relevant prognostic biomarker in the management of gastric cancer. Most studies have shown a correlation between advanced pathological features, such as tumour size, invasion and metastatic spread, and expression of CSC markers. In addition, CD44 and CD133 expression was found to be an independent predictor of poorer disease free survival (in resected gastric cancer) and overall survival (see Table 3). These findings support the preclinical evidence of CD44 and CD133 as CSC markers, as patients with tumours expressing these markers would be expected to have a poorer prognosis due to the CSC traits of chemoradioresistance, increased tumourigenicity and metastatic potential.

CD44 is expressed on up to 80% of primary gastric cancer resection specimens[145] and is associated with more advanced clinicopathological features and poorer prognosis (Table 3). A meta-analysis which included 18 studies examining CD44 expression in gastric cancer, although limited by significant methodological flaws, including no qualitative analysis of included studies, and considerable heterogeneity in pooled result, found statistically significant associations with advanced tumour stage (pooled odds ratio (OR) = 2.05, 95% confidence interval (CI): 1.12-3.75, $P = 0.02$), tumor size (pooled OR = 1.42, 95% CI: 1.08-1.87, $P = 0.01$), and lymph node (LN) metastasis (pooled OR = 1.50, 95% CI: 1.14-1.98, $P = 0.004$) [146]. Although four studies have shown CD44+ expression to be an independent predictor of survival, it is important to note that the two largest case series did not show an impact of CD44 expression on overall survival [147,148]. The heterogeneity in results is likely due to variation in experimental procedures and patient populations.

Despite the contradictory pre-clinical data, CD133 role as a CSC marker is supported by numerous immunohistochemical studies in resected primary gastric cancer which show a

consistent association with numerous high risk clinicopathological features, and independent correlation with poorer disease free and overall survival (Table 3). A recent meta-analysis found strong evidence that CD133 expression in resected gastric cancer was associated with poorer 5 year overall survival (OR= 0.2, 95% CI 0.14-0.29, $p<0.00001$), although it should be noted that all included studies were conducted in Asian populations, limiting the applicability to Western patients [149]. Furthermore, a recent study which detected circulating CSCs using CD133 and ABCG2 as markers, found that the presence of peripheral blood CD133+ cells correlated with a poorer prognosis, and isolated CD133+/ABCG2+ cells were able to be passaged in mouse models and showed self-renewal, heterogeneous linage and increased tumourigenicity [150].

CD44 variant expression appears to be more specific for malignant tissue. Expression of CD44 variants, including v5, v6 and v9, in resected gastric cancer is associated with adverse clinical outcomes including worse overall survival, more advanced tumours and lymphovascular invasion. A meta-analysis found CD44v6 expression was related with LN metastasis (pooled OR = 2.26, 95% CI: 1.40-3.64, $P = 0.0008$), lymphatic invasion (pooled OR = 1.45, 95% CI: 1.05-2.01, $P = 0.02$) , and venous invasion (pooled OR = 1.62, 95% CI: 1.20-2.18, $P = 0.001$) , but not tumour stage (pooled OR = 0.68, 95% CI: 0.36-1.28, $P = 0.23$) [146].

ALDH1 expression has been shown to be associated with poor prognostic clinicopathological features in resected primary gastric cancer, although it is not significantly associated with poorer survival [132].

Table 3: Statistically significant clinicopathological and prognostic associations with CD44 and CD133 immunohistochemical expression in gastric cancer.

Association	CD44 References	CD133 References
Intestinal histology	Mayer *et al* 1993 [66] Hong *et al* 1995 [151] Ghaffarzadehgan *et al* 2008 [152] Nosrati *et al* 2014 [153]	Wakamatsu *et al* 2011 [132] Lee *et al* 2012 [154] Nosrati *et al* 2014 [153]
Higher TNM stage	Wakamatsu *et al* 2012 [132] Chen *et al* 2013 [67]	Yu *et al* 2010 [155] Zhao *et al* 2010 [156] Hashimoto *et al* 2014 [157] Chen *et al* 2013 [67]
Larger tumour size / Deeper invasion	Nosrati *et al* 2014 [153]	Yu *et al* 2010 [155] Zhao *et al* 2010 [156] Lee *et al* 2012 [154] Chen *et al* 2013 [67]
Lymphovascular invasion	Nosrati *et al* 2014 [153]	Lee *et al* 2012 [154]
Higher grade / Poorer differentiation	Wang *et al* 2011 [145] Chen *et al* 2013 [67]	Zhao *et al* 2010 [156] Jiang *et al* 2012 [158] Hashimoto *et al* 2014 [157]
Presence of distant metastasis	Mayer *et al* 1993 [66] Chen *et al* 2013 [67]	Chen *et al* 2013 [67] Hashimoto *et al* 2014 [157]
Positive lymph nodes	Wakamatsu *et al* 2012 [132]	Yu *et al* 2010 [155] Zhao *et al* 2010 [156] Wakamatsu *et al* 2011 [132] Hashimoto *et al* 2014 [157]
Poorer disease free survival (multivariate)	Mayer *et al* 1993 [66]	Lee *et al* 2012 [154] Hashimoto *et al* 2014 [157]
Poorer overall survival (multivariate)	Mayer *et al* 1993 [66] Ghaffarzadehgan *et al* 2008 [152] Wakamatsu *et al* 2012 [132] Chen *et al* 2013 [67]	Yu *et al* 2010 [155] Zhao *et al* 2010 [156] Wang *et al* 2011 [145] Lee *et al* 2012 [154] Chen *et al* 2013 [67] Hashimoto *et al* 2014 [157] Wakamatsu *et al* 2011 [132]

1.2.2 Targeting CSCs in gastric cancer

The CSC model has important clinical implications for cancer treatment. There is strong evidence that CSCs are resistant to traditional chemotherapy and radiotherapy, and are enriched in the residual tumour after these treatments [45,119,123,159]. The CSCs subsequently renew the tumour bulk with the development of treatment resistant clones. Consequently, a specific and efficacious CSC targeted treatment is required for the complete elimination of a cancer. These targeted treatments should be administered in conjunction with conventional chemotherapy/radiotherapy to reduce the tumour bulk and minimise the risk of differentiated cancer cells acquiring CSC-like properties [40].

1.2.2.1 Targeting cancer stem cell surface markers

One proposed method of CSC specific treatment is drugs targeted at CSC surface markers. As discussed above, there are significant challenges with this approach due to the widespread expression of these markers on non-malignant tissue, and the rarity of CSCs in the tumour. Although there is promising data emerging from the preclinical setting targeting CD44, CD133, EpCam and CD90 (discussed below), the largest hurdle will be demonstrating safety and efficacy *in vivo.*

Methodologies targeting CD44 include anti-CD44 monoclonal antibodies [160], and anti-CD44 antibody or aptamer labelled liposomes [161,162]. The CD44 ligand, hyaluronic acid, has also been used to label nanocarriers and conjugates, with demonstrated efficacy in reducing CD44+ cells in pancreatic [163] and gastric cancer [164,165]. Although these studies are promising in demonstrating reduction in CSC populations, the clinical utility of these agents may be limited by off-target toxicities [166,167].

Similarly, CD133 has been successfully targeted in preclinical models by anti-CD133 antibody-cytotoxic conjugates in breast [168], ovarian [169], hepatocellular and gastric cancer [170]. Smith *et al* developed a CD133-cytotoxic conjugate which inhibited growth of gastric cancer cell lines *in vitro* [170].

The anti-EpCam antibody MT201 has shown tumour suppression in preclinical studies in prostate and colon cancer, and has advanced to human trials [170,171]. A phase I study has shown reasonable tolerability in combination with chemotherapy in heavily pre-treated breast cancer, with further studies evaluating efficacy underway [172].

It may also be possible to reduce the CSC population through indirect targeting. Jiang *et al*

noted candidate CSC marker CD90 correlated with HER2 expression in gastric cancer [140].While chemotherapy enriched the CD90+ population in primary cancer culture, a combination of chemotherapy and anti-HER2 treatment (trastuzumab) significantly reduced the CD90+ population and prevented tumour growth [140]. The mechanism of this result remains unclear, but suggests an interesting hypothesis to explain why some breast cancer patients with normal HER2 gene expression benefit from trastuzumab [173].

1.2.2.2 Targeting the cancer stem cell signalling pathways

Aberrations in important normal stem cell signalling pathways, such as Hedgehog (HH), Notch, and Wnt, result in the formation of CSCs [47]. These pathways have been shown to be important potential targets for treating CSCs [174].

The Wnt/β-catenin pathway has a physiological role in balancing proliferation, differentiation, and "stemness" of cells, with over-activation leading to tumourigenesis [175]. It is an important pathway in CSC regulation, and many of the cell surface markers discussed above, including CD44, CD24, EpCam, are Wnt targets [176]. Blockage of the Wnt pathways reduces the self-renewal capacity of gastric cancer tumourspheres [177]. Gastric cancer cells overexpressing Wnt-1 resulted in larger mouse xenograft tumours, with increased expression of CSC markers such as CD44, compared to control cells [178]. When salinomycin was used to suppress Wnt and β-catenin expression, the tumours were smaller with reduced CSC populations [178]. Another group disrupted Wnt signalling in CD44+ selected gastric cancer cells using a Wnt-1 antagonist (Dickkopf-1) delivered by adenovirus serotype 5, inhibiting cancer cell survival, colony formation and invasion [179]. These agents are awaiting clinical validation.

Aberrant activation of the HH pathway causes neoplastic transformation in a variety of tumours including gastric cancer [180]. HH signalling maintains the CSC phenotype, and *in vitro* targeting of the HH pathway decreases the tumourigenicity and invasion capability of gastric cancer spheroids [123], and reverses chemoresistance [127]. Yoon *et al* [123] retrospectively performed immunohistochemistry on gastric cancer samples from a negative randomised phase II trial examining the addition of a HH inhibitor (vismodegib) to chemotherapy in gastric cancer, and found that there was improved survival in patients expressing CSC markers (CD44). This exciting finding is the first evidence of CSC expression as a predictive biomarker in gastric cancer, and demonstrates the immediate clinical applicability of targeting CSC pathways as an adjunct to chemotherapy.

Notch signalling is important in gastric epithelial stem cell homeostasis, and is implicated in

gastric cancer tumourigenesis [181-183]. Gamma-Secretase inhibitors which block the Notch pathway reduce CSC markers and cancer growth [184]. They are currently in early clinical trials, but may be limited by toxicity due to their non-specific activity.

1.2.3 Urokinase Plasminogen activation (uPA) system and gastro-oesophageal cancer

1.2.3.1 The uPA system and malignancy

A key process in the development and progression of cancer, including establishment of metastatic disease, is the invasion of malignant cells into normal tissue. This complex process relies on tumour-associated proteolysis, resulting in the breakdown of extracellular matrix (ECM) and basement membranes barriers [185]. The plasminogen activation system is critical for tumour associated proteolysis [186,187]. Two distinct serine protease plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) activate the circulating zymogen plasminogen to the broad spectrum serine protease plasmin, which has a well-defined role in fibrinolysis of clots [186,188]. While tPA is primarily associated with vascular fibrinolysis, uPA has a more defined role in tissue degradation as well as extravascular fibrinolysis and is thus considered to be responsible for most of the activated plasminogen associated with cancer invasion and metastasis [186,189,190].

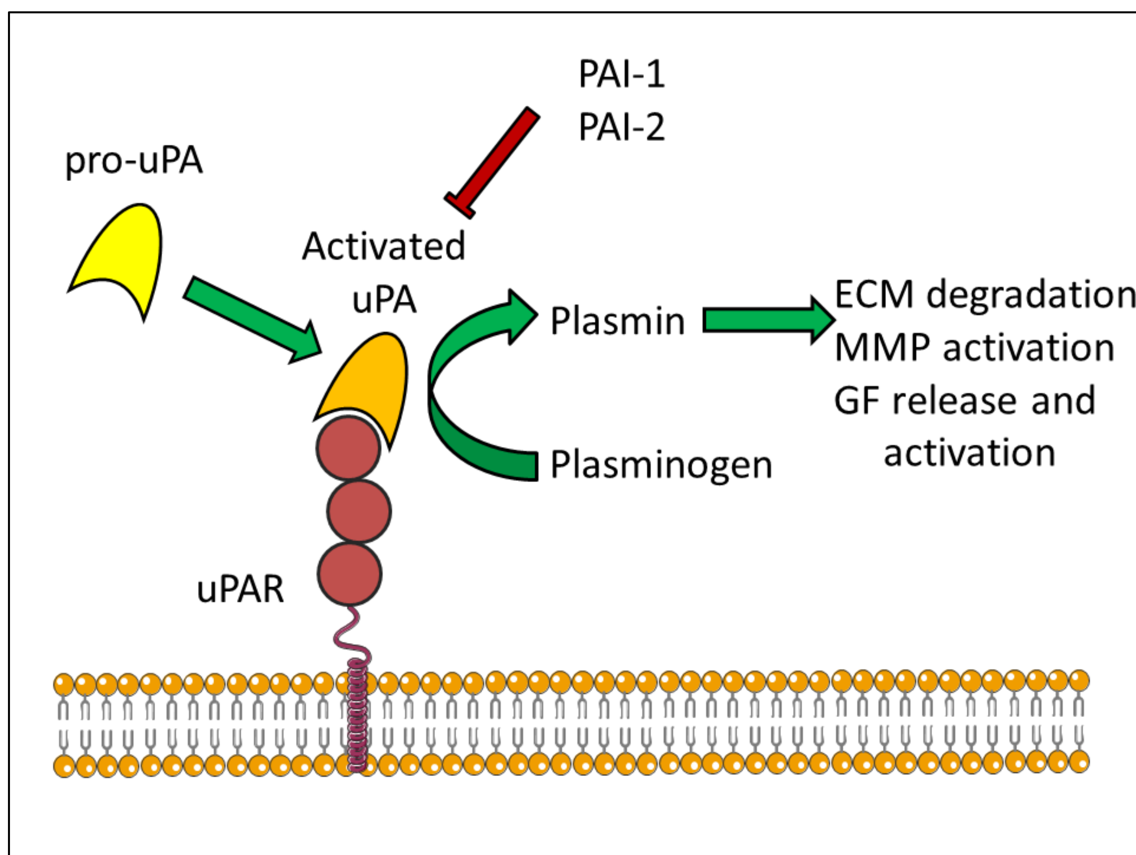

Figure 3: Schematic representation of the urokinase plasminogen activation (uPA) system. The membrane bound urokinase receptor (uPAR) binds circulating inactive pro-uPA, facilitating the activation of pro-uPA to uPA which subsequently converts cell surface co-localised plasminogen to plasmin that can directly degrade components of the extracellular matrix (ECM) and activate pro-matrix metalloproteases (MMP) to further break down ECM. Plasminogen activator inhibitors 1 or 2 (PAI-1, PAI-2) are efficient endogenous inhibitors of uPA.

The uPA protein is secreted as a zymogen (pro-urokinase), and is activated after it binds with very high affinity to its specific cell surface receptor, uPAR, through cleavage of the uPA Lys_{158}-Ile_{159} peptide bond by various proteases, including plasmin [186,191]. Once activated, the disulphide-bonded two-chain uPA catalyses the activation of plasmin from co-localised plasminogen, which in turn directly degrades components of the ECM, and promotes further degradation and tissue remodelling by activating pro-metalloproteinases (MMPs) and by releasing, thus activating, latent growth factors from the ECM [185,190]. MMP-2 is overexpressed in uPA/uPAR positive gastric cancer, suggesting that the MMP and uPA system cooperate during tumour invasion [192]. uPA has reported uPAR independent roles, including mitogentic effects [193], and proteolysis of plasmin to allow cellular migration [194]. As uPAR increases the plasminogen activator activity of uPA several hundred fold, the majority of the uPA effect was

traditionally thought to be uPAR dependent [190]. However, this paradigm has been challenged following a recent study which demonstrated that the development of metastases was dependent on uPA, but not uPAR, in a murine transgenic MMTV-PyMT breast cancer model [195].
The uPAR protein has a glycosyl phosphatidyl anchor attaching it to the plasma membrane [196,197]. Invasion of cancer cells into lymphvascular spaces is facilitated by the expression of uPAR on the invasive front of a tumour [198,199]. This may be by focussing uPA, hence plasmin activity, at the cell surface and/or through complex direct and indirect interactions with a range of binding partners (including vitronectin, integrins, growth factor receptors, and others) [200,201], through which uPAR affects cellular migration, angiogenesis, regulating cAMP levels, and is thus essential for the intravasation of blood vessels [196,202,203]. uPAR expression may represent a suitable marker for early detection of the onset of invasion for both gastrointestinal and breast cancers, expressed only on invasive carcinomas but not premalignant states such as Barrett's oesophagus or carcinoma in situ [204,205].

uPAis efficiently inhibited by two subtypes of serpin (serine proteinase inhibitor) family members, plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and -2 (PAI-2 /SerpinB2) which have disparate roles in cancer growth and metastasis. PAI-1 is considered to be the major inhibitor of the uPA system, forming a covalent complex with active uPA bound to uPAR, leading to the internalisation of the entire complex via the Low Density Lipoprotein related protein-1 and other endocytosis receptors of the LDLR family [206] . Following lysosome degradation of the complex, uPAR is recycled back to the cell surface [207]. Although believed to have a physiological role as an inhibitor of the uPA system, PAI-1 has a paradoxical protumourigenic role, increasing tumour invasion and angiogenesis, and correlated with poor prognosis [208]. Cancer cell models suggest that a critical balance of both uPA and PAI-1 is required for invasion [209].

The role of PAI-2 in cancer is less clear. Although both PAIs mediate uPA/uPAR endocytosis in an LDLR dependant process, the uPA-PAI-2 complex interacts with these endocytosis receptors with different binding kinetics to those of uPA:PAI-1 and without stimulating intracellular signalling events over and above that of uPA binding to uPAR [210-212]. PAI-2 expression is increased in tumour compared to normal tissue, perhaps due to a host inflammatory response, but high stromal expression is associated with prolonged (in contrast to the other components of the uPA system) survival in breast cancer [191].

The uPA system is expressed on both cancer cells and the supporting stroma [188]. In one of the original in-situ hybridization studies in colon cancer, uPA and uPAR were found to be mainly expressed on the stromal and tumour cells, respectively, at the invasive front of a tumour,

facilitating proteolysis required for invasion [213]. While immunohistochemical studies in gastric cancer have shown stromal uPA/uPAR expression, higher expression is seen on tumour cells, and it is postulated that this tumour cell specific uPA/uPAR explains the aggressive biology exhibited by these cancers, and is more relevant for prognostic outcomes [214-216].

Expression of the uPA system has been shown to be an important prognostic marker in a variety of cancers including breast cancer[217-219], lung cancer [220], and colorectal cancer [221], with the combination of uPA and PAI-1 expression recommended to be incorporated into routine clinical care of node negative breast cancer by the American Society of Clinical Oncologists[222,223].

1.2.3.2 The uPA system and gastroesophageal cancer

The prognostic role of expression of the uPA system in gastroesophageal cancer is not clear. While numerous studies have been performed to investigate this association, the studies have employed differing methodology and included different patient populations, leading to contrasting results. Conclusions from a previous meta-analysis are limited by significant methodological flaws and the pooled analysis of gastric and colon cancer [224].

1.2.4 Circulating tumour cells

As cancers form and grow, tumours cells are shed from the tumour mass into the circulation, where they can be detected in blood samples. Study of these circulating tumour cells (CTCs) is a rapidly developing field in oncology research. There is an increasing recognition of the limitations of using primary tumour features to guide systemic cancer treatment, due to tumour heterogeneity and the frequent disparity observed between primary and metastatic sites [225]. Metastatic biopsies are rarely undertaken however, due to both inaccessibility of metastatic sites and procedure morbidity. CTC sampling therefore provides a 'real time' view of the cancer using only peripheral blood samples, avoiding the need of repeat invasive biopsies [226].

1.2.4.1 Techniques for isolating CTCs – technical challenges

As CTCs occur at very low concentrations in the peripheral blood (ranging 1-10 cells per 10 ml of blood in most cancer patients) there are considerable technical challenges in developing robust detection protocols[226].

Most CTC detection protocols require two steps. Initially the peripheral blood is enriched for CTCs using physical properties (such as ficoll density gradient) or expression of cell surface markers, using either positive selection or negative depletion. For example, the CellSearch system (Menarini-Silicon Biosystems, Huntington Valley, PA, USA)- the only current FDA approved CTC system, uses positive immunomagnetic isolation of EpCAM (an epithelial cell marker) positive cells using anti human EpCAM antibody conjugated magnetic beads [227]. The CellSearch system immunoprobes the cells enriched by EpCAM targeting for cytokeratins (a second epithelial marker to improve specificity), DAPI (nuclear stain), and excludes CD45 (a leukocyte marker) positive cells. As such, CTCs are defined as EpCAM/cytokeratin(CK)/DAPI positive and CD45 negative cells isolated from a blood sample. CTCs enumerated using this approach have been shown to be clinically relevant prognostic biomarkers in a range of cancers including breast [228], bowel [229], and prostate cancer [230]. Other strategies of CTC enrichment rely on physical differences of tumour cells versus blood cells, mainly size (filter enrichment of cells larger than most blood cells) or the plasticity of cells. Nevertheless, these methods still need CTC identification by usually immunostaining to verify the nature of CTCs versus residual blood cells. CTC isolation approaches have been thoroughly reviewed by Alix-Panabières *et al* [222].

Each approach to CTC enrichment has potential advantages and drawbacks. Positive selection using techniques such as immunomagnetic separation based on cell molecular expression (eg with EpCAM in the CellSearch System) is quite specific, reproducible and fully validated in

regards to correlation of detected CTC numbers to clinical parameters. It is limited by the fact that CTCs have to express the isolation target (usually EpCAM) and the identification marker (usually cytokeratin). Overall the technology has relatively low sensitivity, high costs, and further analysis of the enriched CTCs is often dependent on further purification of single or pooled CTCs. In contrast, density gradient (eg Ficoll, OncoQUick) and size based filtrations are simple, cheaper, but also limited by CTC molecular phenotype establishment using immunostaining (usually EpCAM and/or cytokeratin probing). The advantages of some methods are that they allow for easier subsequent analysis of CTCs. However they are limited by poor enrichment and low specificity/sensitivity [231].

Similarly each detection method of CTCs within the enriched specimen has different strengths. A cytometric approach allows the user to assess to some degree cell morphology and other characteristics for enumeration and molecular characterisation. However ideal CTC markers have yet to be identified, and there is the potential for lower sensitivity. Nucleic acid based (RT-qPCR) techniques are antibody independent, highly sensitive and allow for multimarker assays, but are limited by high false positive rates and the user is unable to isolate or assess CTCs. There is therefore a need to identify new CTC biomarkers in order to overcome limitations of the current approach.

1.2.4.2 EpCAM based CTC capture and enumeration in gastroesophageal adenocarcinoma:

Enumeration of CTCs has been confirmed as a clinically relevant prognostic marker in gastroesophageal cancer. The strongest evidence is from studies with EpCAM based capture using the CellSearch platform, the most commonly used approach (studies summarised in Table 4).

Table 4: CTC studies using EpCAM based capture (CellSearch platform) in gastroesophageal cancers including resectable gastric cancer (RGC), advanced gastric cancer (AGC), resectable esophageal cancer (REC), and advanced gastroesophageal cancer (AGOC).

Study	**Patient Population**	**Collection time points**	**Positive cutoff CTC/7.5ml**	**Summary of results**
Allard *et al* 2004 [227]	9 AGC	Prior to treatment	≥2	Detection rate 31%
Hiraiwa *et al* 2008 [232]	17 RGC 27 AGC	Prior to treatment	≥2	Healthy volunteers 0% Non metastatic: 14.3% Metastatic 55.6%
Matasusaka *et al* 2010 [233]	52 AGC	Prior to tx, 2wks, 4 wks	>4	Detected in 33% of cases CTC levels at 2 and 4 weeks predictive of response and prognostic
Uenosone *et al* 2013 [234]	148 RGC 103 AGC	Prior to treatment	≥ 1	No CTC detected in healthy volunteers 11.3% in RGC 60.2% in AGC Average 3.5 CTCs / 7.5ml Poorer DFS and OS with positive CTCs
Sclafani *et al* 2014 [235]	18 AGC	Prior to treatment	≥2	44% at baseline Small numbers, closure of commercial support
Lee *et al* 2015 [236]	100 AGC	Prior to treatment	≥5	Detection rate 28% Poorer OS and PFS with positive CTC
Reeh *et al* 2015 [237]	68 REC (adeno)	Prior to treatment	≥1	Detection rate 20.6% Poorer RFS and OS with positive CTCs
Okabe *et al* 2015 [238]	25 AGC	Prior to treatment	≥1	Detection rate 18.4% Poorer PFS with positive CTCs
Li *et al* 2016[239]	136 AGC	Prior to tx and 6 weeks	≥3	Positive CTCs after 6 weeks associated with poor PFS, response rate, and OS
Pernot *et al* 2017 [240]	106 AGOC	Prior to treatment and day 28	≥2	Poorer DFS and OS with detectable CTCs CTCs at day 28 predictive of disease control

These studies confirm the validity of EpCAM capture for enumeration of CTCs in gastroesophageal cancer and demonstrate the following results: CTCs were detected in 11.6 – 60.2% of patients with gastroesophageal cancer; detectable and higher numbers of CTCs more often occurs in patients with advanced stages of disease [238]; higher CTC counts correlate with worse clinicopathological features such as tumour size/invasion, lymphovascular invasion and lymph node status[234,238]; similar to other solid tumours, a positive CTC count is an independent risk factor associated with poorer clinical outcomes[233,234,236-239]; and dynamic changes in CTCs with treatment may provide an early prediction of response[238-240]. A subsequent meta-analysis of the above CTC studies confirm these findings [241].

Despite the utility of EpCAM, a major challenge in the use of CTCs as biomarkers is the development of an ideal marker to detect the rare cancer cells in the large numbers of benign cells – that is – a marker that is *always* and *only* expressed on malignant cells [226]. Single marker, such as CK expression alone, has high false positive results (from 20 to 50%), which is reduced by using a second marker [242]. Moreover, there are likely heterogeneous subpopulations of CTCs which have differing malignant potential. For example, EpCAM and CK are downregulated as cells undergo epithelial-mesenchymal transition (EMT), and consequently methods using EpCAM capture do not detect CTCs in the EMT phenotype [243]. Although confirmed as prognostic, CTC enumeration using current markers has more limited success in guiding treatments [244]. Furthermore, modelling studies suggest that 1 in 60 million CTCs form viable metastases [245], and there have been studies reporting long term (>22years) persisting CTCs with no clinically visible disease [246]. Therefore there is a need to develop novel CTCs markers, beyond the currently utilised EpCAM/CK markers, to identify CTCs responsible for metastatic disease. CSC biomarkers and the uPA system hold potential for the detection of CTCs beyond traditional EpCAM methods.

1.2.4.3 CSC markers and CTCs

As discussed in section 1.2.1, CSC are the subpopulation of cancer cells that are capable of initiating tumour growth and therefore responsible for the initiation and propagation of metastatic disease. CTCs are therefore likely to be enriched for CSCs, and CSC markers may provide an improved means for detecting biologically relevant CTCs [247]. Several small studies have explored the role of CSC markers in CTCs in gastroesophageal cancer using CD44 and CD133. Li *et al* showed CK/CD44 positive cells within the peripheral blood mononuclear cell (PMBC) layer was prognostic in patients with gastric cancer [73]. Yuan *et al* used CD45 depletion combined with CD44 positive selection to isolate tumourigenic CTCs [248]. The CD44+ fraction of EpCAM+ cells in peripheral blood samples isolated by flow cytometry shows correlation with disease stage and venous invasion, as well as response to chemotherapy, while the CD44-

fraction did not [249]. The number of CD133+ cells isolated in peripheral blood samples was significantly associated with clinical outcomes for patients with gastric cancer [150]. So far, no study has compared the utility of CSC markers to identify CTCs to the widely accepted EpCAM+/CK+/DAPI+/CD45- phenotype.
As indicated above, one important limitation of this approach is the widespread expression of CD44 and CD133 on non-malignant tissue. For example, CD44 is expressed on many cells found in the circulation including leukocytes and endothelial cells[250], and may reduce specificity of CTC detection, thus a combination strategy probing for EpCAM/CK/CD45 plus CD44 and CD133 maybe a viable method.

1.2.4.4 CTCs and the uPA system

The uPA system is the key proteolytic pathway to facilitate invasion of cancer cells into stromal tissue. uPAR has been identified as an important marker on CTCs in breast cancer, with uPAR+ CTCs enriched for stem cell pathways, and able to adhere, proliferate and invade [251]. While there are no studies examining expression of the uPA system in gastroesophageal cancer CTCs, there are some compelling results supporting the importance of uPA system in disseminated tumour cells (DTC) in bone marrow. DTCs are thought to represent the fraction of CTCs capable of entering distant sites as the first step in establishing metastases [252]. Allgayer *et al* used CK18 to identify DTC in the bone marrow in 156 patients who had undergone a curative resection for localised gastric cancer. They found while overall CK18+ DTC was not associated with prognosis, the CK18+/uPAR+ subset was, suggesting uPAR identifies the critical subpopulation of cells responsible for establishment of metastasis [253,254].

1.2.4.5 Circulating tumour cell culture

Research into the mechanism of metastasis initiation, formation, and propagation has been hampered by limited access to cancer cells within the various stages of the metastatic cascade. CTCs provide a unique window into the biology of cancer as it spreads through the blood stream. As CTCs are very rare cells and few CTCs are isolated (often 1 – 10 cells per 10 ml of blood) by current methodologies, this research has been hampered by the low number of cells available for analysis. CTC culture provides an expanded cell population for expression analysis, functional assays, and drug sensitivity [255,256]. Moreover, long term primary CTC cultures provide a laboratory tool for the investigation of the biology of metastasis formation [257]. However establishment of long term primary CTC cell cultures has proven to be challenging. Despite intensive efforts, only four long term CTCs cultures have been established and reported in the literature [258-261], with no reported success in gastroesophageal cancer (Table 5).

Table 5: Long term CTC cell lines established worldwide

ULA – Ultra low attachment; Ab/Am – Antibiotic/Antimycotic; EGF – epithelial growth factor; FGF – fibroblast growth factor

Study	Cancer	Isolation Method	Media / culture conditions	Culture success rate (successful cultures/all patients)
Yu *et al* 2014[259]	Breast	20ml EDTA CTC-iChip	RPMI 1640 EGF 20ng/ml FGF 20ng/ml B27 1x Ab/Am Hypoxic incubator ULA plate	6/36
Cayrefourcq *et al* 2015[258]	Colorectal	10ml EDTA Stemcell RosetteSep CD45 depletion	DMEM/F12 , Insulin 20ug/ml, 1% N2, EGF 20ng/ml, L-Gutamine 2mM FGF2 10ng/ml, 2% FCS 24 well ULA plate,	1/71
Zhang *et al* 2013[261]	Breast	20 – 45ml	*Medium 1 (1wk)* DMEM/F12, Insulin 5mg/ml Hydrocort 0.5mg/ml, EGF 20ng/ml, FGF-2 20ng/ml *Medium 2 (D7-D22)* EpiCult-C with 10% FBS and 1% P/S, T75 flask *Medium 3 (>22d)* DMEM/F12 , 10% FCS, 1%P/S	3/8
Gao *et al* 2014[260]	Prostate	8ml EDTA Stemcell RosetteSep CD45 depletion Seeded in growth factor reduced matrigrel	*Complex media (see supp materials)* DMEM/F12 EGF 50ng/ml FGF10 10ng/ml FGF2 1ng/ml Testosterone, nicotinamide R-spondin 1 B27 Glutamx HEPES Primocin	1/100

Short term cultures have also been developed in a variety of tumours. One group has used the MetaCell assay and RPMI-1640 supplemented with fetal bovine serum to establish short term (<14 days) CTC cultures in prostate, pancreatic, oesophageal, gastric and bladder cancer [262-265]. The EPISPOT assay detects cytokeratin secretion by CTCs during short term culture following CD45 depletion as an enrichment step [266,267], and the Vitatex (Vitatex Inc, Stony Brook NY, USA) detects the invasion ability of CTCs through a fluorescent matrix [268].

An alternative approach is to expand the CTC population by injecting them into immunodeficient mice. This approach has been used successfully in breast cancer [269], although required >1000 CTCs/7.5 ml blood, a far higher number than is seen in most patients. A CTC xenograft model has also been demonstrated in gastric cancer. The CD45 negative fraction of peripheral blood mononuclear cells from patients with gastric cancer formed tumour like structures in nine immunodeficient mice. One of the nine tumours expressed gastric epithelial markers and CSC markers, providing proof of concept of the xenograft as a functional CTC assay [270].

1.3 Clinical Biomarkers in Colon Cancer

A key challenge in modern clinical oncology is the integration of diverse types of prognostic information to provide an accurate, but highly individualised, estimate of prognosis. Despite intensive efforts and hundreds of publications, there are very few biomarkers that are currently routinely incorporated into the clinical care of patients with colon cancer [271]. While there is emerging evidence for many molecular markers, including microRNA, circulating tumour DNA, tumour suppressor genes, and molecular signatures such as the Oncotype DX (Genomic Health Inc, Redwood City, CA, USA) colon cancer assay, however these await further study and external validation prior to clinical use. Despite initial promise, subsequent studies on the same or related markers have demonstrated inconsistent or contradictory results [271].

As noted above, the most important prognostic factor in colon cancer is pathological stage at diagnosis [25]. Other validated, clinically utilised, prognostic factors include lymphovascular and perineural invasion [272-274], histological grade [275], and CEA level [276-278]. Important molecular biomarkers include microsatellite instability (MSI) [279], RAS and BRAF mutations [280-282], and more recently, the consensus molecular subtype [283], although MSI is the only molecular biomarker routinely used in early stage colon cancer in Australia.

The consensus molecular subtype (CMS, Figure 4), developed through an international collaboration to resolve inconsistencies in molecular profiling between independent groups, is

the most widely adopted approach to classify colorectal cancer into distinct molecular subtypes with significant biological differences[283]. The CMS proposes 4 distinct molecular subtypes. CMS1 (MSI-like) include hypermutated and microsatellite instable tumours, and are enriched for CIMP and BRAF mutations. CMS2 (canonical) are tumours with chromosomal instability and marked Wnt and MYC activation. CMS3 (metabolic) demonstrate disruption of metabolic pathways and are enriched for KRAS mutations. CMS4 (mesenchymal) show prominent stromal invasion, angiogenesis and a mesenchymal phenotype[283]. Importantly the CMS has shown significant prognostic associations, with CMS4 tumours associated with the poorest survival[284]. Although it is hoped that the CMS will facilitate molecular guidance for individuation of treatments, the large number of genes included in the CMS has prevented widespread adoption in routine clinical care[285]

CMS1 MSI Immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGFβ activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 4: The consensus molecular subtype (CMS) outlining key features and proportion of the four subtypes of colorectal cancer. Taken from Guinney et al[283]

Clinical factors are known to act as surrogates for tumour biology. For example, Asian female patients with advanced non-small cell lung cancer are enriched for the targetable EGFR mutation [286]. Identifying key clinical factors in colon cancer may not only identify prognostic markers, but also provide insight into the underlying pathobiology.

1.3.1 Primary tumour location in colon cancer

There is an increasing interest in identifying the differences between right sided (RsCC) and left sided colon cancer (LsCC), with a growing body of evidence to suggest that right sided colon cancers follow a different disease process compared to left sided tumours. The proximal and distal colons are physiologically separate, arising from distinct embryological origins, with differences in tumour genetics, histology, presentation, and clinical features [287-289]. Patients with

right colon cancer are older, more likely to be female, have more comorbidities, with poorer tumour histopathological features [290-293]. Epidemiological studies suggest a recent shift from right to left sided colon cancer [294]. RsCCs are more likely to have adverse histological features, higher rates of BRAF mutations and MSI, and demonstrate a molecular profile distinct from LsCC [283,287,295-297].

Despite this, there is ongoing debate whether primary tumour location is an independent prognostic factor in colon cancer. Most, but not all studies have found poorer survival with right colon cancer [292,293,298-300]. Tumour stage may play a role, with a large Surveillance, Epidemiology, and End Results (SEER) program study showing worse overall survival in Stage III RsCC patients, but not in Stage I or II [292], although these finding have been recently challenged by a propensity score matched analysis of the SEER database, which showed a better prognosis in RsCC patients [298]. Further studies are required to elucidate the differences between LsCC and RsCC, particularly in locoregional disease.

1.3.2 Age as a predictor of benefit to adjuvant chemotherapy

Adjuvant (post operative) chemotherapy is an essential component of treatment in stage III and high risk stage II colon cancers. The fluoropyrimidines are the most efficacious single agent in CRC and are commonly utilised as the backbone in combination treatments. Oral fluropyrimidines (capecitabine) are as efficacious as intravenous (fluorouracil; 5-FU with modulating leucovorin) with an improved safety profile [301,302]. Large randomised control trials establish fluoropyrimidine based chemotherapy as the standard of care in the adjuvant setting, with pooled analysis including 3302 patients with stage II and III colorectal cancer showing a 30% reduction in risk of recurrence and 26% decreased risk of death with fluourouracil based adjuvant chemotherapy [303]. The MOSAIC trial demonstrated an additional 23% DFS and 20% OS benefit by adding oxaliplatin to 5-FU in patients with stage III CRC [304], with a similar result seen in the NSABP C-07 trial [305].

Despite the above evidence, the optimum regimen for elderly patients remains uncertain. As only a minority of patients in clinical trials are older than 70 years, the efficacy and safety of adjuvant chemotherapy with an oxaliplatin doublet in elderly patients is unclear. For example, in the adjuvant colon cancer end points database (ACCENT) which includes individual patient data from 14500 participants in 18 fluoropyrimidine - based adjuvant trials, only 18% are older than 70 years [306].

Currently available trial data for adjuvant chemotherapy in elderly population is conflicting. Subgroup analyses from the pivotal phase III MOSAIC and NSABP C-07 trials show a survival

benefit only in patients <70 years [304,305]. Similarly, there was no DFS) or OS improvement with the addition of oxaliplatin in the 2575 patients ≥70 years in the ACCENT database [306]. In contrast however, pooled individual patient data from 904 patients ≥70 years from the NSABP C-08, XELOXA, X-ACT, and AVANT studies showed an attenuated, but statistically significant benefit to the addition of oxaliplatin, including those with comorbidities [307].

Similarly, retrospective patient series demonstrate contrasting results. While the largest series, drawn from multiple USA databases including the SEER database, found a statistically significant benefit to adjuvant oxaliplatin in elderly patients (70-74 years old) and those with comorbidities [308], this was less clear in patients >75 [309] and was not seen in other, smaller studies [310,311].

As a consequence of these uncertainties, current guidelines recommend discussing incorporation of oxaliplatin with patients over 70 years based on individual circumstances, although fluoropyrimidine monotherapy is an appropriate choice for adjuvant therapy in the elderly [18,312,313].

1.4 Aims and Objectives

It is clear that cancer biomarkers are critically important in clinical oncology. In addition to providing valuable prognostic information, biomarkers assist in patient risk assessment, prediction of response to treatment, and monitoring progress of disease, all key factors in improving the individualisation and delivery of treatment. Furthermore, biomarkers provide insight into the mechanisms of cancer and identify novel targets for therapeutic agents. Despite improvements in systemic treatments, prognosis remains poor for the majority of patients with gastroesophageal cancer. This is largely due to the lack of robust biomarkers available in gastroesophageal cancer to guide systemic treatment.

The overall aim of this thesis was to identify clinical and molecular biomarkers of gastrointestinal cancer to ultimately improve delivery of treatment to patients with these diseases.

The specific aims of this work were to:

i) Determine if expression of the urokinase plasminogen activator (uPA) system and cancer stem cell (CSC) markers are biomarkers in gastroesophageal cancer

ii) Develop and validate a protocol for the cryopreservation and thawing of samples for circulating tumour cell (CTC) analysis

iii) Determine if uPA receptor (uPAR) expression improves the prognostic value of circulating tumour cells above currently employed markers

iv) Establish primary cancer cell cultures from circulating tumours cells isolated from patients with gastroesophageal cancer

v) Explore primary tumour location as biomarker in locoregional colon cancer.

vi) Investigate the suitability of age as a predictive determinate of benefit to oxaliplatin-based adjuvant chemotherapy in locoregional colon cancer

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Chapter 2

Expression of the uPA system in resectable gastroesophageal cancer

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TB, MC, KV, MA – interpretation of results, manuscript revisions

MR – concept development, interpretation of results, manuscript draft and revisions

Expression of the urokinase plasminogen activation system in primary gastro-oesophageal cancer: A systematic review and meta-analysis.

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Abstract

Background: The urokinase plasminogen activation (uPA) system is a crucial pathway for tumour invasion and establishment of metastasis. Although there is good evidence that uPA system expression is a clinically relevant biomarker in some solid tumours, its role in gastroesophageal cancer is uncertain.

Methods: We undertook a systematic review evaluating expression of uPA, urokinase plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and plasminogen activator inhibitor-2 (PAI-2/SerpinB2) on primary oesophageal, gastro-oesophageal junction, and gastric adenocarcinomas. We performed a meta-analysis of clinicopathological associations, overall survival (OS) and recurrence free survival (RFS).

Results: We identified 41 studies encompassing 2689 patients which fulfilled the inclusion criteria. uPA, uPAR, or PAI-1 expression is significantly associated with high risk clinicopathological features. High uPA expression is associated with a shorter RFS (HR 1.90 95% 1.16 – 3.11, p=0.01) and OS (HR 2.21 95% CI 1.74 – 2.80, p<0.0001). High uPAR expression is associated with poorer OS (HR 2.21 95%CI 1.82 – 2.69, p<0.0001). High PAI-1 expression is associated with shorter RFS (HR 1.96 96% CI 1.07 – 3.58, p=0.03) and OS (HR 1.84 95%CI 1.28 – 2.64, p<0.0001). There was no significant association between PAI-2 expression and OS (HR 0.97 95%CI 0.48 – 1.94, p<0.92) although data was limited.

Conclusion: We conclude that the uPA system is a clinically relevant biomarker in primary gastroesophageal cancer, with higher expression of uPA, uPAR and PAI-1 associated with higher risk disease and poorer prognosis. This also highlights the potential utility of the uPA system as a therapeutic target for improved treatment strategies.

2.1 Introduction

Gastroesophageal cancer is a common and lethal malignancy, marked by modest response to systemic therapies[1]. A deeper understanding of molecular events characterising carcinogenesis, invasion, progression and metastasis is central for the development of novel therapies.

2.1.1 The uPA system

A key process in the development and progression of cancer, including establishment of metastatic disease, is the invasion of malignant cells into normal tissue. The plasminogen activation system, particularly the urokinase-type plasminogen activator (uPA) system, is critical for tumour-associated proteolysis to breakdown extracellular matrix (ECM) and basement membranes barriers[2,3]. The uPA system has a defined role in tissue degradation and extravascular fibrinolysis, and is responsible for most of the activated plasminogen associated with cancer invasion and metastasis[3,4] (Figure 1).

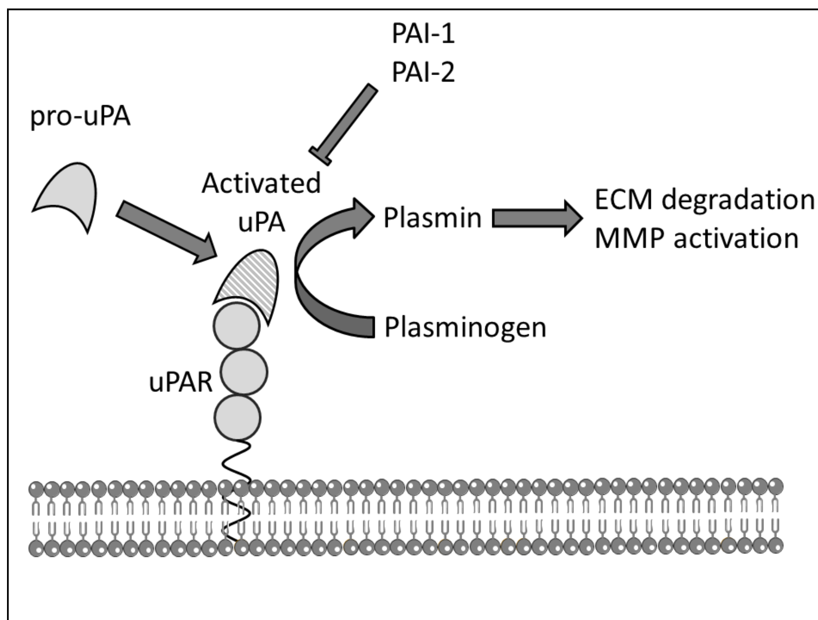

Figure 1: The uPA system: Schematic representation of the urokinase plasminogen activation (uPA) system. The membrane bound urokinase receptor (uPAR) binds circulating inactive pro-uPA, facilitating the activation of pro-uPA to uPA which subsequently converts co-localised plasminogen to plasmin that can directly degrade components of the extracellular matrix (ECM) and activate pro-matrix metalloproteases (MMP) to further break down ECM. Plasminogen activator inhibitors 1 or 2 (PAI-1, PAI-2) are efficient endogenous inhibitors of uPA.

The uPA protein is secreted as a zymogen and activated on high affinity binding to its specific cell surface receptor uPAR. Once activated, uPA catalyses the activation of co-localised plasminogen to plasmin, which in turn directly degrades components of the ECM, and promotes further degradation and tissue remodelling by activating pro-metalloproteinases and by releasing, thus activating, latent growth factors from the ECM[2,4].

The uPA receptor (uPAR) is anchored to the plasma membrane, localising the uPA system to the cell surface [5]. High expression of uPAR on the invasive front of tumours facilitates invasion and other roles in cellular migration and angiogenesis [6,7]. uPAR expression may be a suitable marker for the onset of invasion of both gastro-intestinal and breast cancer as it is expressed only on invasive carcinomas, not premalignant states such as Barrett's oesophagus [8].

Urokinase-type plasminogen activator is efficiently inhibited by two subtypes of serpin (serine proteinase inhibitor) family members, plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and -2 (PAI-2 /SerpinB2). Both form a covalent complex with uPA/uPAR leading to internalisation of the entire complex [9] . Although believed to have a physiological role as an inhibitor of the uPA system, PAI-1 has a paradoxical protumourgenic role, increasing tumour invasion and angiogenesis, and correlated with poor prognosis [10]. The role of PAI-2 in cancer is less clear. Although both PAIs mediate uPA/uPAR endocytosis, the uPA-PAI-2 complex interacts with endocytosis receptors with different binding kinetics to those of uPA: PAI-1 and without stimulating intracellular signalling events over and above that of uPA binding to uPAR [11].

While the uPA system is expressed on both cancer cells and the supporting stroma, higher expression is seen on tumour cells, and is postulated that the tumour cell specific uPA/uPAR explains the aggressive biology exhibited by these cancers, and is more relevant for prognostic outcomes [12-15]. Expression of the uPA system has been shown to be an important prognostic marker in a variety of cancers including breast cancer[16], lung cancer[17], and colorectal cancer[18], with the combination of uPA and PAI-1 expression recommended to be incorporated into routine clinical care of node negative breast cancer[19].

In this study we aim to perform a comprehensive systematic review of expression of the uPA system encompassing uPA, uPAR, PAI-1, and PAI-2 in primary, resectable gastro-oesophageal cancer, and undertake meta-analyses of prognostic outcomes (recurrence free survival and overall survival), and association with relevant clinicopathological variables. To the best of our knowledge, this is the first meta-analysis to examine and compare the expression of these key components of uPA system in primary gastro-oesophageal cancer.

2.2 Methods

Methods are reported according to Preferred Reporting for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[20].

2.2.1 Study eligibility/selection criteria

We included all studies which examined the following components of the urokinase plasminogen activation system uPA, uPAR, PAI-1 or PAI-2, in resected primary esophageal, gastroesophageal junction, or gastric adenocarcinomas. Other tumour pathologies were excluded. For inclusion in the quantitative synthesis, studies were required to report the association of the following outcomes with uPA system expression: overall survival (OS), recurrence-free survival (RFS), or clinicopathological variables.

Two authors (DB, JC) independently performed the search and screened the studies. The primary outcome was OS; secondary outcomes were RFS, and correlation of clinicopathological variables with uPA system expression.

2.2.2 Study search strategy

We searched the following databases in February 2015 for all trials fulfilling the above criteria: Medline (1950 – present); EMBASE (1966 – present); Cochrane Central Register of Controlled Trials, and Cochrane Database of Systematic Reviews; PubMed.

To maximize sensitivity the following search terms were used: Stomach Neoplasms (MESH) OR Esophageal neoplasms (MESH) OR Gastrointestinal neoplasms (MESH) OR Gastric cancer.mp OR Gastric carcinoma.mp OR esophageal cancer.mp OR oesophageal cancer.mp OR gastroesophageal cancer.mp AND Receptors, urokinase plasminogen activator (MESH) OR Urokinase-type plasminogen activator (MESH) OR Plasminogen activator inhibitor 1 (MESH) OR Plasminogen activator inhibitor.mp OR PAI-1.mp OR PAI-2.mp OR Urokinase* plasminogen.mp OR uPA*.mp. Reference lists of included studies and review articles were hand searched. The search was restricted to studies published in English.

2.2.3 Data collection

Study data was independently collected by two authors (DB, JC) using standardized electronic data collection forms. The following was collected for each study: patient number, primary tumour location (gastric/oesophageal/COJ), cancer stage, treatment received by patient; uPA components assessed (uPA, uPAR, PAI-1, PAI-2) and method, patient followup; outcomes (OS or RFS), clinicopathological correlations (including TMN stage, tumour grade, lymphatic invasion, vascular

invasion). For studies which used IHC, expression analysis was restricted to tumour cells only (stromal expression was not included in the meta-analysis). Any disagreement was resolved with consensus by a third author (MR)

2.2.4 Assessment of bias within studies

All studies included in the meta-analyses were assessed for bias using the Quality In Prognosis Studies (QUIPS) tool which assesses for potential sources of bias in six domains namely: study participation; study attrition and loss to followup; prognostic factor measurement; outcome measurement; study confounding; and statistical analysis and reporting[21].

2.2.5 Statistical analysis

We extracted the hazard ratio (HR) and their 95% confidence intervals (CI) for time-to-event outcomes including RFS and OS. If both univariate and multivariate HR were published the univariate results were preferentially used. Where no HR was provided in published data, it was estimated from available results or Kaplan-Meier survival curves using previously described methods[22].
HRs were synthesized using the generic inverse variance method and a random effect model using RevMan5.1 analysis software. Statistical heterogeneity was assessed using the I^2 statistic. We performed pre-specified subgroup analysis for overall survival for: primary location (gastric or oesophageal), cancer cell specific expression (using IHC) compared to whole cell lysis (using RT-PCR/ELISA).
Clinicopathological associations were summarized using odds ratios (OR) derived from published results. This analysis was limited to studies using IHC, as other methods presented expression results as means, rather than percentage of patients expressing. Expression rates were described with mean and range, and compared using the student's t-test.

2.3 Results

2.3.1 Included studies

The trial flow is provided in Figure 2. We identified 267 reports matching criteria for inclusion in the study, of which 109 were selected for abstract review, and 60 subsequently for full text review. Forty one studies (including 2689 patients) fulfilled criteria for inclusion in the systematic review. Of these, 22 studies (1966 patients) provided sufficient data for inclusion in the formal quantitative meta-analysis: 19 studies were excluded for the following reasons: 13 studies did not examine prognostic or clinicopathological associations[23-34], 3 reports were matched case control studies[35-37], and 4 studies reported insufficient published data to derive a HR[38-42].

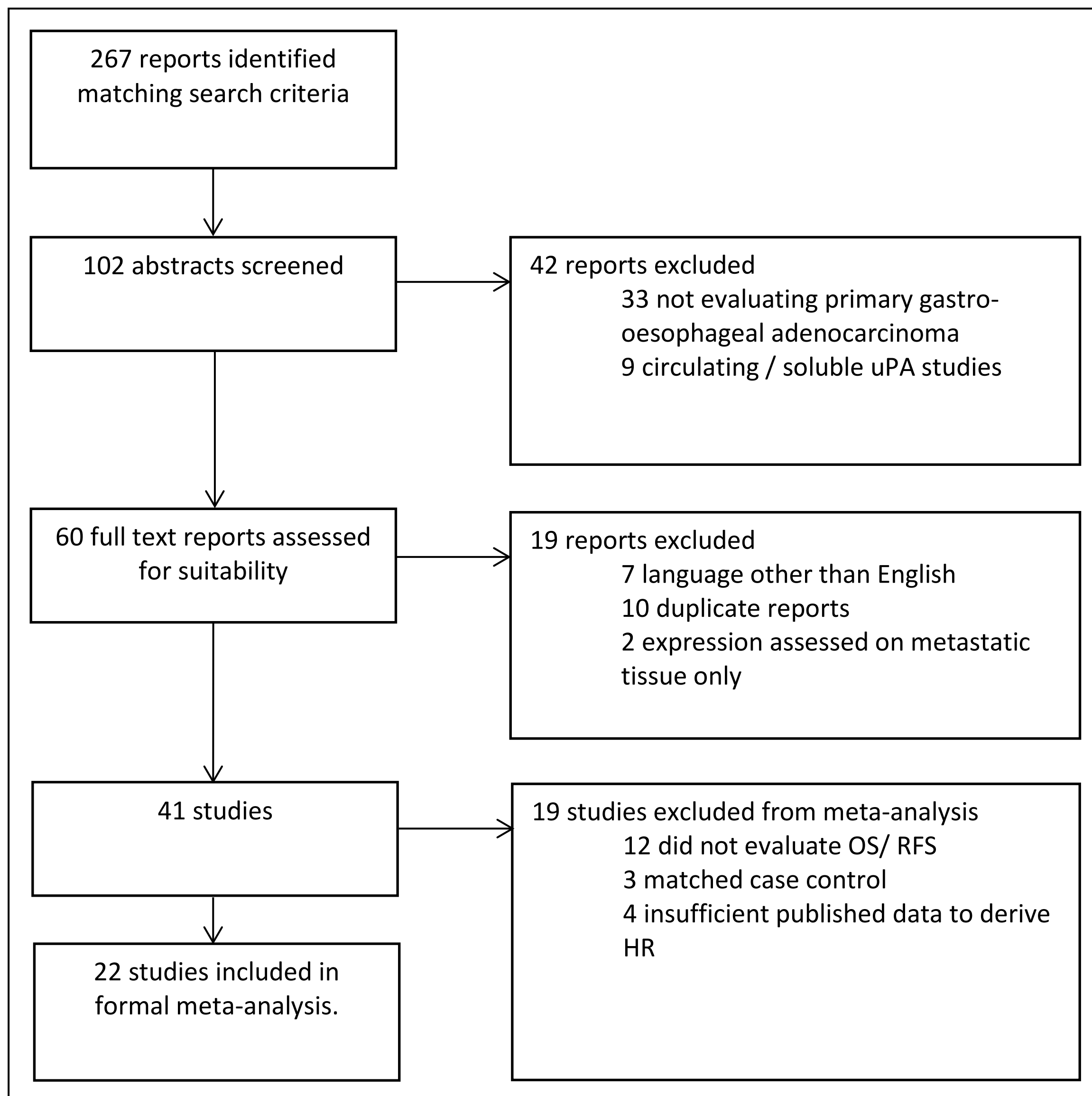

Figure 2: Study selection flow diagram. HR –hazard ratio; OS – overall survival; RFS – recurrence free survival.

The characteristics of the included studies are summarized in Supplementary Table 1. Eighteen studies evaluated uPA system expression in gastric cancer (1732 patients), one study included oesophageal, junctional and gastric cancers (39 patients), and two studies examined oesophageal cancer only (105 patients). Expression of the uPA system was assessed using immunohistochemistry (IHC, 12 studies, 1273 patients), enzyme-linked immunosorbent assay (ELISA, 5 studies, 344 patients), reverse transcription polymerase chain reaction (RT-PCR, 3 studies, 153 patients), or in-situ hybridisation (ISH, one study, 105 patients).

Hazard ratios directly extracted for 3 studies [8,12,43]. The multivariate HR was used when univariate

value was not provided [43]. When only subgroup outcome data (tumour core or peripheral zone) were available, the results for peripheral "invasion" zone were used [8,12]. Hazard ratios were estimated for the remaining studies using published data. 4 studies reported a "non-statistically significant OS" result for uPA system expression, but did not publish sufficient data for inclusion in meta-analysis [39-42].

2.3.2 Bias risk

The risk of bias summary is summarized in Figure 3. Only 4 studies [43-46] were deemed low risk in all bias domains. Fourteen studies did not clearly define the study population [8,13,14,47-57] and 11 studies did not report completeness of followup [8,13,14,47-50,53,55,56,58]. Most studies adequately reported method of measurement of the uPA system, although 5 studies did not report whether there was a second independent reviewer or blinding to clinical information[14,52,56,57,59]. The follow-up protocol was underreported in 14 studies [8,12-14,47-53,55-57], although this is unlikely to bias the results for overall survival analyses. Most studies did not report details of the surgical, medical, or radiation treatments received by patients, and were Urokinase plasminogen activator (uPA)

	Bias Domain					
Study	Study Participation	Study Attrition	Prognostic factor Measurement	Outcome Measurement	Study Confounding	Statistical analysis and reporting
Allott 2012						
Alpizar Alpizar 2012						
Bayer 2006						
Cho 1997						
Ganesh 1996						
Heiss 1995						
Ito 1996						
Kaneko 2003						
Kawasaki 1998						
Laerum 2012						
Lee 2004						
Luebke 2006						
Maeda 1996						
Murata 1998						
Nekarda 1994						
Nekarda 1998						
Okusa 1999						
Plebani 1997						
Sakakibara 2006						
Taniguchi 1998						
Yonemura 1997						
Zhang 2006						

Figure 3: Risk of bias summary. For each bias domain: green = "low risk" means that sufficient data was available to allow assessment of quality and fulfilled criteria for each domain, and accordingly is deemed low risk of bias. Orange = "unclear risk" means that insufficient data was presented to adequately assess the quality of the domain and accordingly the study has potentially high risk of bias. There were no studies deemed high risk of bias.

2.3.3 uPA system expression rates

The expression rates are summarized in table 1. There was no significant difference in expression rates seen between cell specific (IHC, ISH) or whole tissue lysate (RT-PCR, ELISA).

	Number of Studies (number of patients)	Mean expression IHC/ISH (%)	Mean expression ELISA/RT-PCR (%)	p value (students t-test)
uPA	19 (1629)	59.5	42.6	0.1
uPAR	15 (1352)	55.1	56.7	0.4
PAI-1	15 (1337)	63.7	42.9	0.1
PAI-2	3 (300)	43	-	-

Table 1: Expression of the uPA system in gastroesophageal cancer . IHC – immunohistochemistry, IHC – immunohistochemistry, RT-PCT - reverse transcription polymerase chain reaction; ISH – in-situ hybridisation; ELISA - enzyme-linked immunosorbent assay

2.3.4 Urokinase plasminogen activator (uPA)

2.3.4.1 uPA and clinicopathological associations

uPA expression is significantly associated with poorer clinicopathological features in resected gastroesophageal cancer including: Advanced T stage (T3/4 vs T1/2) (OR 2.79 95% CI 1.80 – 4.32, p<0.0001), nodal metastases (OR 2.30 95% CI 1.63 – 3.51, p<0.0001), liver metastases (RR 6.77 95% CI 2.70 – 16.96, p<0.0001), peritoneal metastases (OR 2.09 95% CI 1.29 – 3.36, p=0.003), lymphatic invasion (OR 2.28 95% CI 1.31 – 3.97, p=0.0003), and vascular invasion (OR=2.43 95% CI 1.53 – 3.86, p=0.0002) (5 studies, 522 patients, supplementary Figure 1). There is no significant association with histology (poorly differentiated vs well differentiated).

2.3.4.2 uPA expression and prognosis

uPA expression was significantly associated with a worse RFS (3 studies, 467 participants, HR 1.90 95% 1.16 – 3.11, p=0.01) (see supplementary Figure 2). There was no significant difference in RFS seen between studies using IHC (HR 1.77) or ELISA (HR 2.36) to assess uPA expression (test for subgroup differences Chi^2=0.37, p=0.54).

uPA expression is significantly associated with poorer OS (12 studies, 1094 participants, HR 2.21 95% CI 1.74 – 2.80, p<0.0001) (see Figure 4). There was no significant difference in OS between studies which used IHC (HR 1.94) or ELISA (HR=2.99) to assess uPA expression (p=0.38). Sensitivity analysis showed similar results when analysis was restricted to gastric cancer only (HR 2.07, p<0.00001).

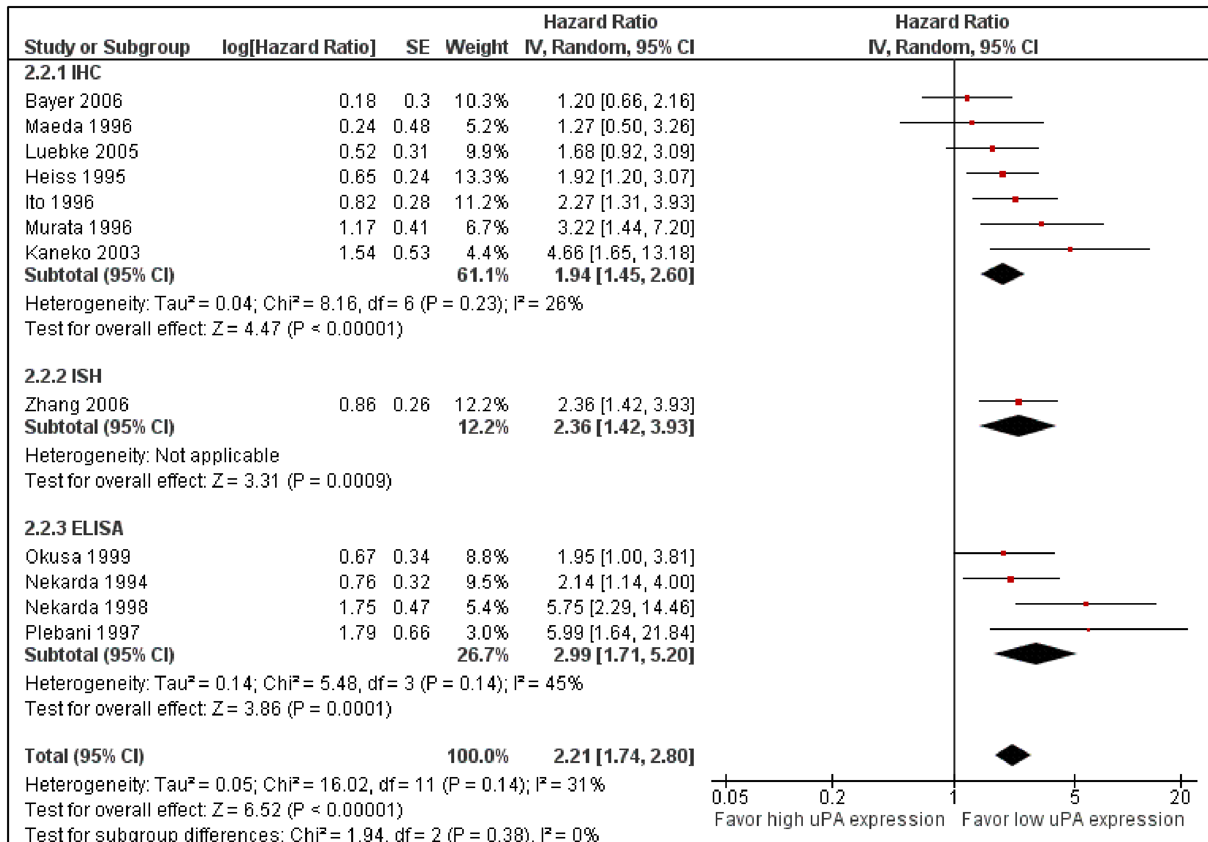

Figure 4: Pooled estimate of hazard ratio (HR) for uPA expression and overall survival (OS). Pooled estimate of hazard ratio (HR) for overall survival. The square on each bar represents the HR for an individual trial, and the bar shows the 95% confidence interval (CI). The diamond represents a pooled estimate with the centre of the diamond giving the HR estimate, and the extremes of the diamond representing the 95% CI.

2.3.5 Urokinase plasminogen activator receptor (uPAR)

2.3.5.1 uPAR expression and clinicopathological associations

uPAR expression on primary resected gastroesophageal cancer is significantly associated with poorer clinicopathological features including: advanced TMN stage (stage III/IV vs I/II, OR 3.41 91% CI 1.55 – 7.53, p=0.002), advanced T stage (OR 2.33 95% CI 1.53 to 3.56, p<0.0001), nodal metastases (OR 2.52 95% CI 1.70 – 3.72, p<0.0001), liver metastases (OR 2.53 95% CI 1.25 – 5.13, p=0.010), peritoneal metastases (OR 3.15 95% CI 1.87 – 5.28, p<0.0001), lymphatic invasion (OR 2.82 95% CI 1.74 – 4.59, p<0.0001) and vascular invasion (OR 3.85 95% CI 2.53 – 5.88, p<0.0001) (six studies, 589 patients, supplementary Figure 3). There is no significant association seen with histology (p=0.6).

2.3.5.2 uPAR expression and prognosis

Only one study provided data for uPAR expression and RFS [59], showing a shorter RFS with uPAR expression (203 patients, HR 2.69, p=0.03).

uPAR expression is associated with poorer OS (11 studies, 1036 patients, HR 2.19 95%CI 1.80 – 2.66, p<0.0001) (Figure 5). There was no significant difference in OS seen between studies which used IHC (HR 2.13), ISH (HR 2.34), ELISA (HR 2.19), or RT-PCR (2.66) to assess uPAR expression (p=0.96).

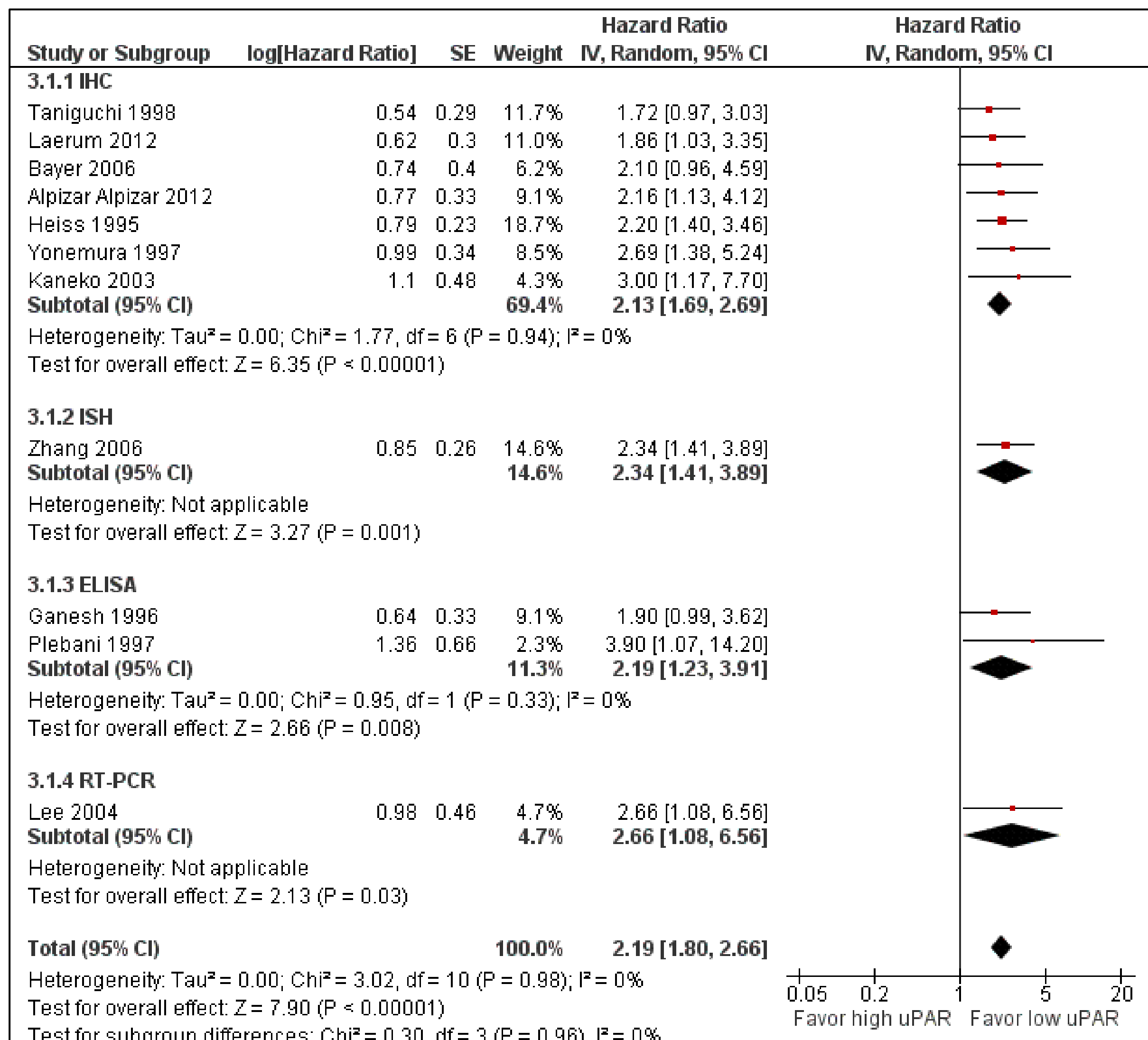

Figure 5: Pooled estimate of hazard ratio (HR) for uPAR expression and overall survival (OS).

2.3.6 Plasminogen Activator Inhibitor-1 (PAI-1)

2.3.6.1 PAI-1 expression and clinicopathological variables

PAI-1 expression on primary resected gastroesophageal cancer is significantly associated with poorer clinicopathological features including: advanced T stage (OR 2.59 95% CI 1.61 to 4.18, p<0.0001), nodal metastases (OR 2.03 95% CI 1.27 – 3.22, p<0.003), lymphatic invasion (OR 2.09 95% CI 1.31 – 3.34, p<0.004) and vascular invasion (OR 1.90 95% CI 1.20 – 3.03, p<0.007) (three studies, 317 patients, supplementary Figure 4). There was no significant association of PAI-1 expression with presence of liver metastases (OR 0.52, p=0.18), peritoneal metastases (OR 1.38, p=0.31), or histology

(OR 0.93, p=0.74).

2.3.6.2 PAI-1 expression and prognosis

PAI-1 expression is associated with shorter RFS (3 studies, 467 patients, HR 1.96 96% CI 1.07 – 3.58, p=0.03) (supplementary Figure 5). There was no significant difference in RFS between studies which used IHC or ELISA to detect PAI-1 expression (p=0.86)

PAI-1 expression is significantly associated with a shorter OS (10 studies, 839 participants, HR 1.84 95%CI 1.28 – 2.64, p<0.0001, Figure 6). Pre-specified subgroup analysis showed a significant difference between studies which assessed PAI-1 expression using IHC (HR 1.20, p=0.47) and ELISA (HR 2.94, p<0.0001) or RT-PCR (HR 2.83, p<0.0001) (p=0.02).

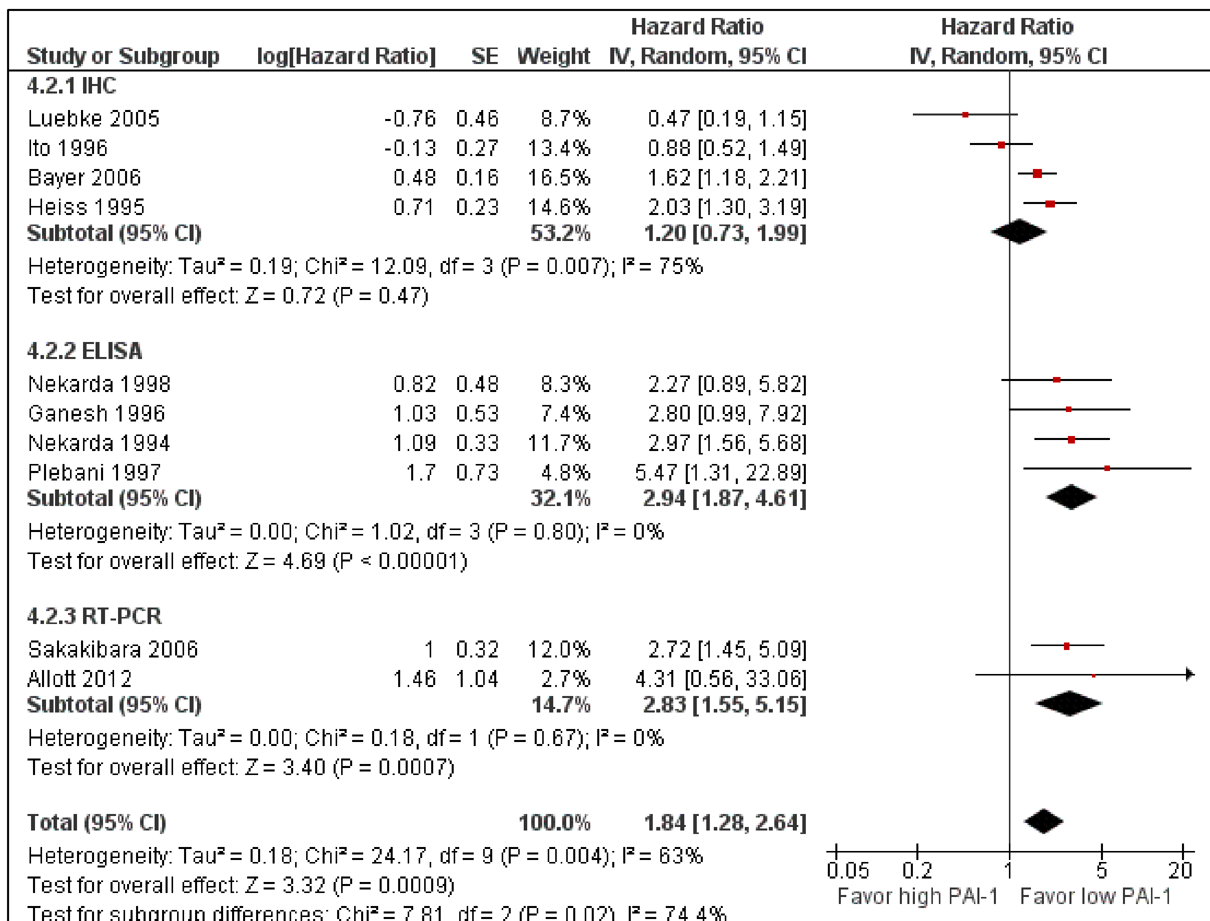

Figure 6: Pooled estimate of hazard ratio (HR) for PAI-1 expression and overall survival (OS).

2.3.7 Plasminogen Activator Inhibitor-2 (PAI-2)

2.3.7.1 PAI-2 expression and clinicopathological variables

There were no studies with sufficient data analyzing PAI-2 expression and clinicopathological variables for inclusion in the meta-analysis.

2.3.7.2 PAI-2 expression and prognosis

No studies published data on PAI-2 expression and RFS. There was no significant association of PAI-2 expression and OS (2 studies, 145 participants, HR 0.97 95%CI 0.48 – 1.94, p<0.92, supplementary Figure 6).

2.3.8 Publication bias

Examination of the funnel plots for the OS analysis for uPA, uPAR and PAI-1 showed asymmetrical plots for all analyses, suggesting absence of smaller negative trials (example plot for uPA provided in supplementary Figure 7).

2.4 Discussion

This meta-analysis confirms the clinical utility of the uPA system as a biomarker in resected gastro-oesophageal adenocarcinoma.

There is good evidence that high expression of uPA, uPAR, and PAI-1 is associated with most high risk clinicopathological features, including advanced T stage, presence of nodal and distant metastases, and lymphovascular invasion, in primary gastro-oesophageal adenocarcinoma. This supports the central role of the uPA system in tumour invasion and metastasis. In contrast, there was no significant association of expression found with poorly differentiated histology, consistent with previously published work which shows that epithelial cell uPA system expression is higher in malignant than benign tissue, but decreases as tumour becomes more poorly differentiated, with a corresponding increase in stromal expression[60].

We also demonstrated that uPA, uPAR, and PAI-1 expression is associated with poorer prognosis in resected gastro-oesophageal cancer, with both a shorter RFS and OS in tumours which expressed these markers. However this result should be interpreted with caution due to the following important limitations in our study.

Firstly, only four of the included studies were deemed low risk for all bias domains as assessed by the QUIPS tool. In particular, most studies did not report the treatments patients received which is an

important potential source of confounding for RFS and OS analyses. Additionally, tumours with higher risk clinicopathologic features could reasonably be expected to be more likely to have received neoadjuvant treatment prior to surgery, which may in turn have impacted on the expression of the uPA system. Despite this, it should be noted that similar results were seen in studies deemed low and high risk of study confounding, and heterogeneity was low in both the uPA and uPAR OS meta-analyses (I^2= 31% and 0% respectively, see Figure 4 and 5).

Secondly, there is evidence of underreporting of non-significant results. This is demonstrated by both the funnel plot, as well the selective reporting of only statistically positive findings from included studies. This important bias will cause an overestimation of the effect of expression.
Thirdly, as demonstrated above, tumours that expressed uPA, uPAR and PAI-1 had higher risk features, and would be expected to recur or progress sooner than tumours that did not. The apparent difference in prognostic outcomes may be due to unequal baseline characteristics of the included participants.

We did not show a significant difference in the prognostic outcomes between studies which used a tumour cell specific technique (e.g. IHC) compared to whole tissue lysates (e.g. RT-PCR, ELISA) for uPA and uPAR. This is consistent with other studies which have shown correlation between IHC score and median ELISA value, and supports the cancer cells as a major source of uPA and uPAR expression in the tumour tissue[61].

In contrast, there was a significant different in the expression methodology subgroups in the analysis for PAI-1 and OS (p=0.02), with a non-significant outcome seen in studies using IHC (HR 1.20, p=0.47), compared to significant results with ELISA (HR 2.94, p<0.0001) and RT-PCR (HR 2.83, p=0.0007). This highlights the importance of the stromal production of PAI-1 within the tumour microenvironment [10], as only methods that took into account both stromal and tumour PAI-1 showed statistically significant prognostic outcomes. It has been postulated that in contrast to uPAR, fibroblasts and endothelial cells provide the major source of PAI-1 within the tumour tissue [62]. It is possible that the PAI-1 detected on the tumour cells by IHC may be explained by internalization and accumulation of stromal produced uPA-PAI-1 complexes mediated by tumour uPAR [63]. No IHC studies examined the association between stromal PAI-1 expression and prognostic outcomes in gastro-oesophageal cancer.

All IHC study results used in the meta-analysis were restricted to tumour cell expression only. Similar to other cancers, uPA system expression was highest at the invasive front of the tumour [8,12,13,48]. Only four studies reported stromal expression of the uPA system [8,12,13,59]. Results were conflicting, with only one study showing a significant association of OS with macrophage uPAR

expression[8].

We were unable to show any significant associations with PAI-2 expression with either clinicopathological features or prognostic outcomes, as available data was much more limited. Similarly only 3 studies examined oesophageal cancer, which limits applicability of our results to this subgroup. Sensitivity analysis did not show a different result when oesophageal cancer was excluded from analysis.

In conclusion, expression of the uPA system is a clinically relevant biomarker in gastroesophageal cancer. There is good evidence to support the association of uPA, uPAR, and PAI-1 expression and high risk clinicopathological features. While we found a statistically significant association between uPAR, uPAR and PAI-1 expression and poorer prognosis, our results are tempered by methodical limitations discussed above. Prospective studies are required to further confirm its role as an independent prognostic marker in this disease.

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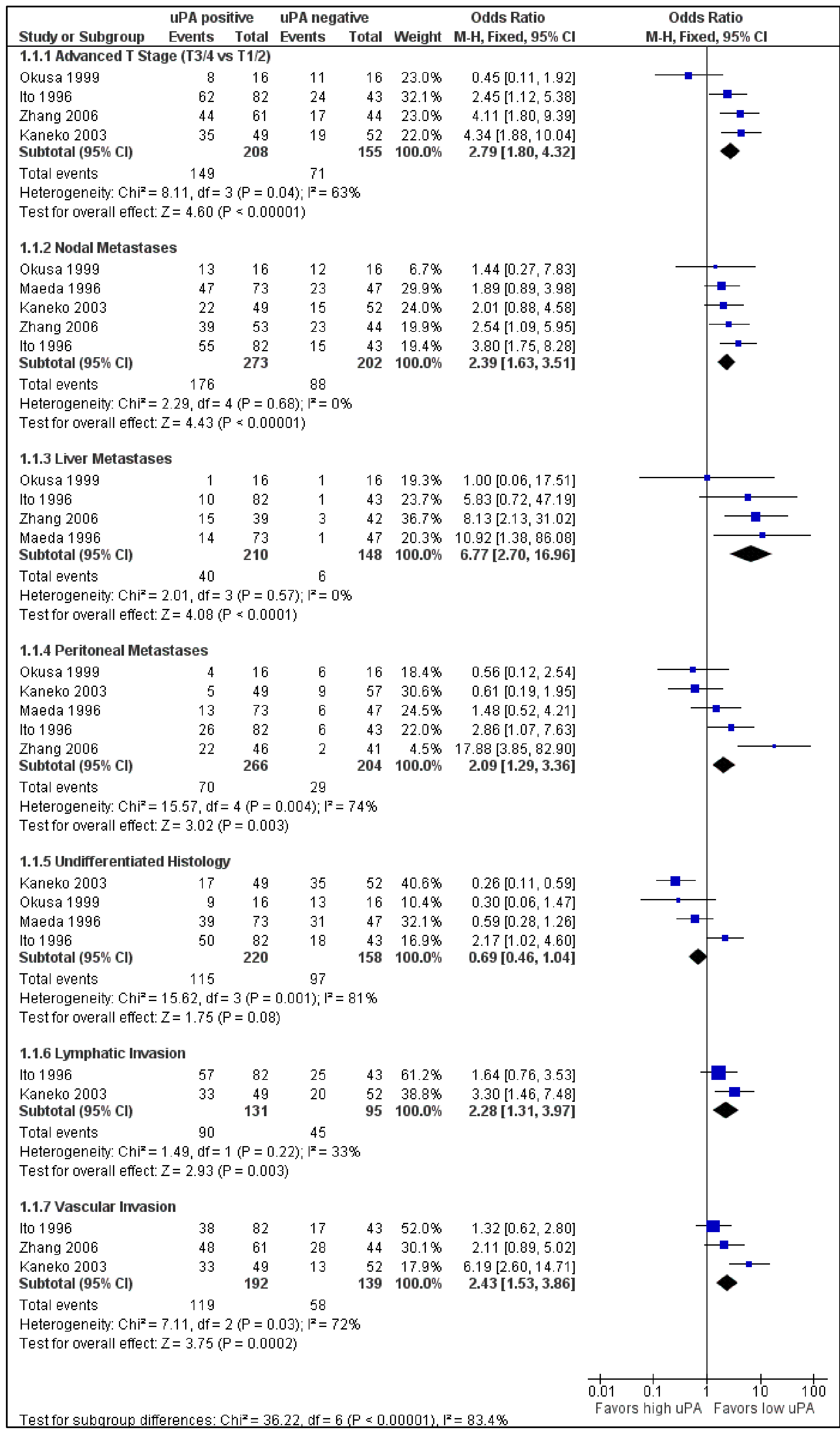
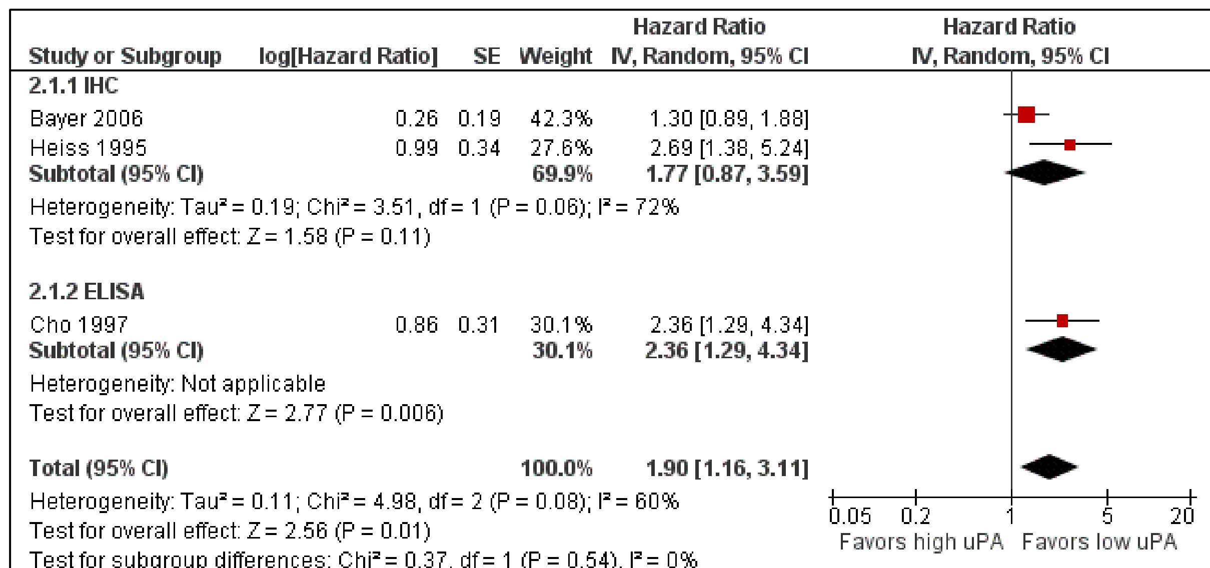
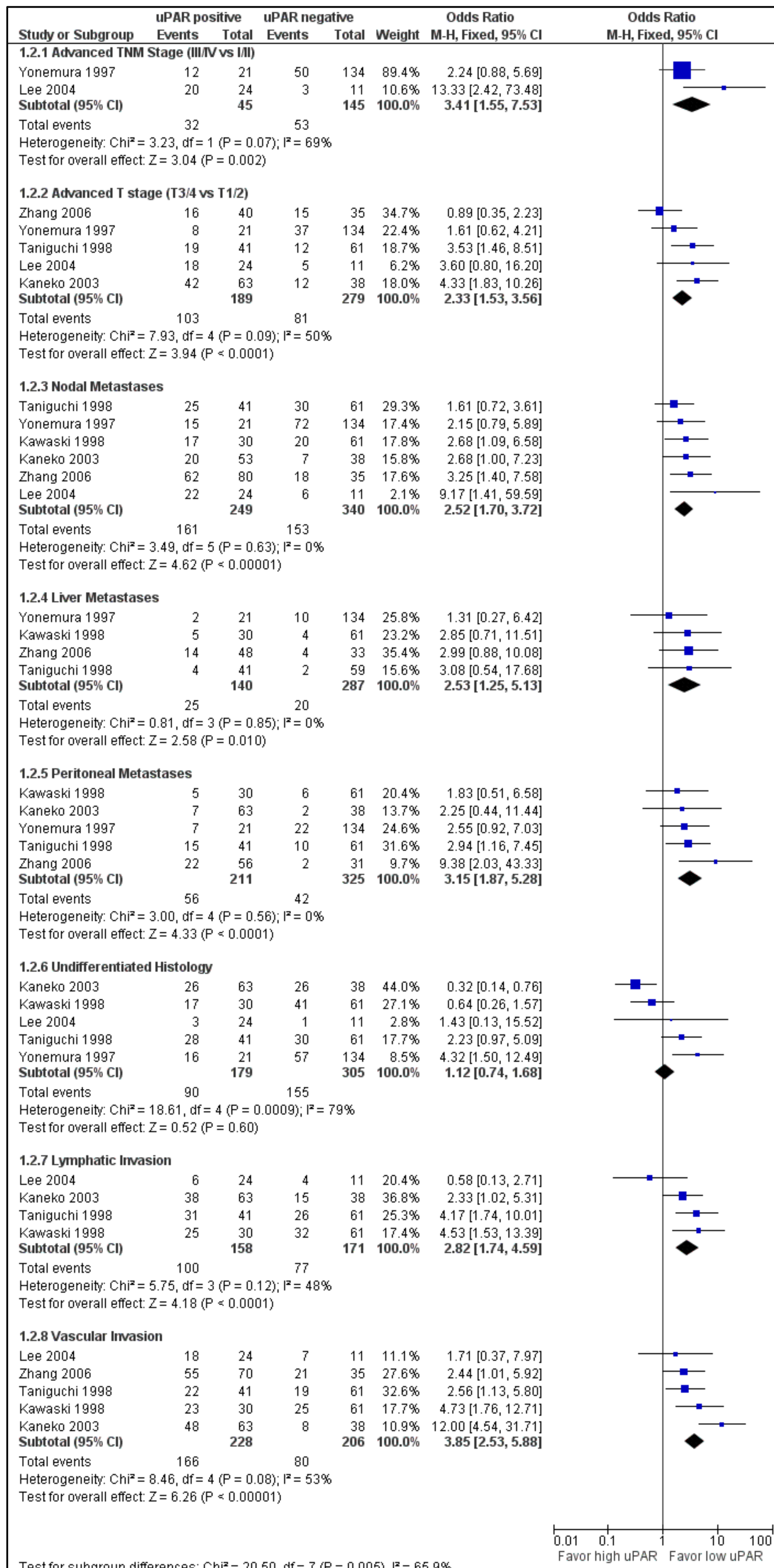
Supplementary Figure 1: Pooled estimate of odds ratio (OR) for uPA expression and clinicopathological factors.



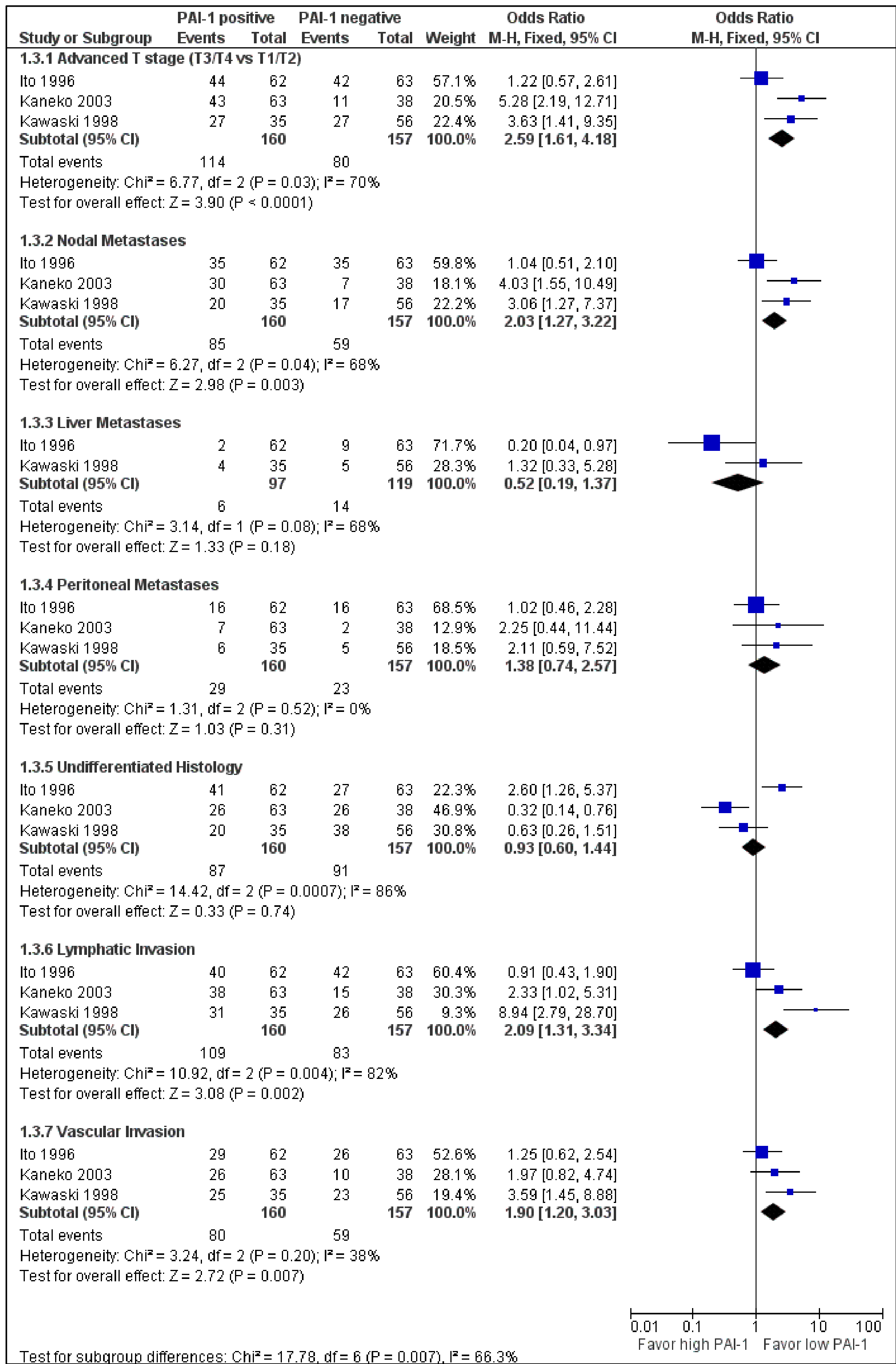
Supplementary Figure 2: Pooled estimate of hazard ratio (HR) for uPA expression and recurrence free survival (RFS).



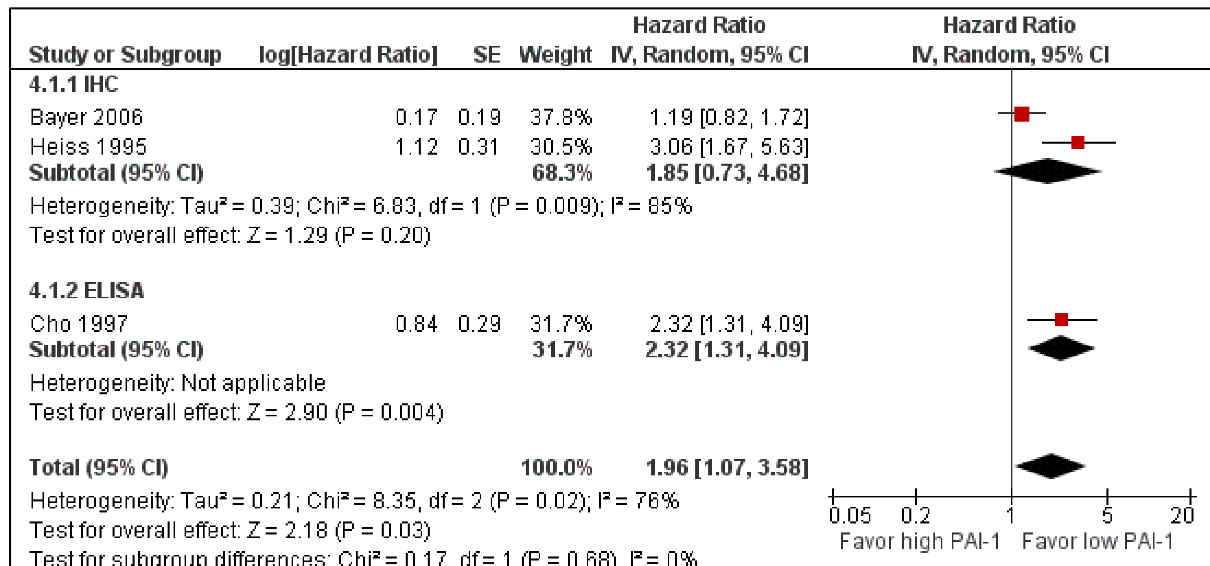
Supplementary Figure 3: Pooled estimate of odds ratio (OR) for uPAR expression and clinicopathological factors.



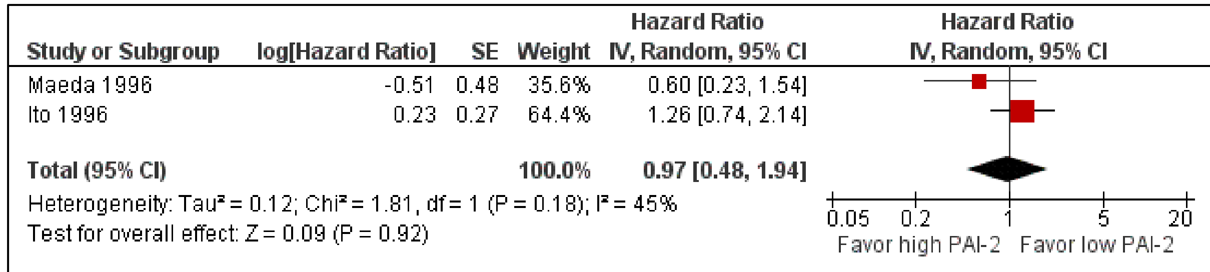
Supplementary Figure 4: Pooled estimate of odds ratio (OR) for PAI-1 expression and clinicopathological factors.



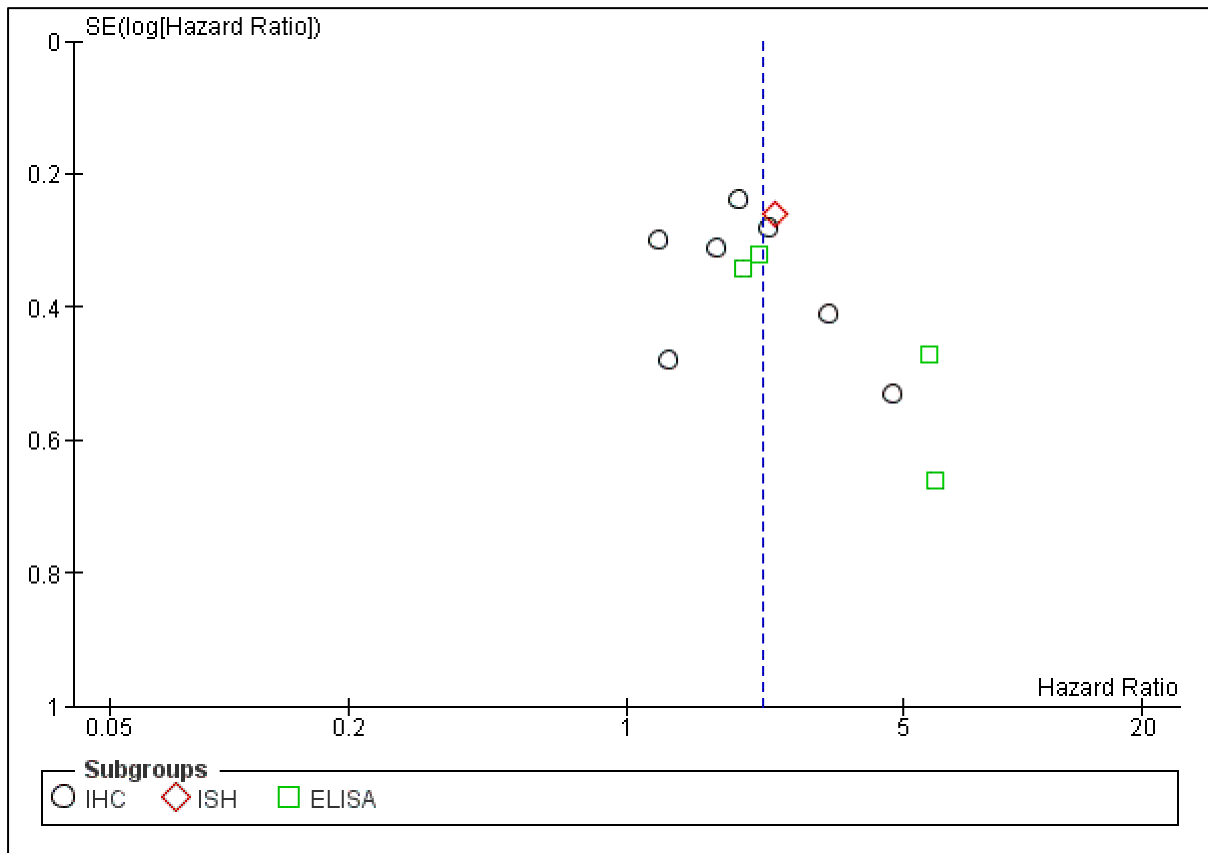
Supplementary Figure 5: Pooled estimate of hazard ratio (HR) for PAI-1 expression and recurrence free survival (RFS).



Supplementary Figure 6: Pooled estimate of hazard ratio (HR) for PAI-2 expression and overall survival (OS).



Supplementary Figure 7: Funnel plot of uPA studies



Supplementary Table 1: Characteristics of included studies.

	Study (year)	Pt No.	Primary location (TNM stage)	Analysis Method	uPA component	Expression Rate (%)
1	Allott (2012) [47]	39	Oesophageal (ND)	RT-PCR	PAI-1	50
2	Alpizar Alpizar (2012) [12]	95	Gastric (I-IV)	IHC	uPAR	90
3	Beyer (2006) [43]	104	Gastric (I-IV)	IHC	uPA	91
					uPAR	88
					PAI-1	90
4	Cho (1997) [44]	160	Gastric (I – IV)	ELISA	uPA	28
					PAI-1	33
5	Ganesh (1996) [46]	50	Gastric (I – IV)	ELISA	uPAR	42
					PAI-1	11
6	Heiss (1995) [59]	203	Gastric (I – IV)	IHC	uPA	75
					uPAR	69
					PAI-1	71
7	Ito (1996) [48]	125	Gastric (ND)	IHC	uPA	66
					PAI-1	50
					PAI-2	52
8	Kaneko (2003) [49]	101	Gastric (ND)	IHC	uPA	23
					uPAR	33
					PAI-1	37
9	Kawasaki (1998) [13]	91	Gastric (I – IV)	IHC	uPAR	33 (IHC)
				ISH		20 (ISH)
					PAI-1	39 (IHC)
						29 (ISH)
10	Lærum (2012) [8]	66	Oesophageal, GOJ, Gastric (I-IV)	IHC	uPAR	90*
11	Lee (2004) [50]	35	Gastric (I – IV)	RT-PCR	uPAR	63
12	Luebke (2006) [58]	105	Gastric (I – IV)	IHC	uPA	84
					PAI-1	93
13	Maeda (1996) [64]	120	Gastric (I – IV)	IHC	uPA	61
					PAI-2	63
14	Murata (1998) [52]	26	Gastric (IV)	IHC	uPA	42
15	Nekarda (1994) [65]	76	Gastric (I – IV)	ELISA	uPA	51
					PAI-1	41
16	Nekarda (1998) [45]	38	Oesophageal (I-IV)	ELISA	uPA	34
					PAI-1	32
17	Okusa (1999) [53]	71	Gastric (I – IV)	ELISA	uPA	50
18	Plebani (1997) [54]	20	Gastric (I – IV)	ELISA	uPA	65
					uPAR	65
					PAI-1	75
19	Sakakibara (2006) [55]	79	Gastric (I – IV)	RT-PCR	PAI-1	58
20	Taniguchi (1998) [56]	102	Gastric (ND)	IHC	uPAR	40
21	Yonemura (1997) [14]	155	Gastric (I – IV)	IHC	uPAR	14
22	Zhang (2006) [57]	105	Gastric (ND)	ISH	uPA	58
					uPAR	67

Pt No – number of patients; uPA – urokinase plasminogen activator; uPAR - urokinase plasminogen activator receptor; PAI-1 – plasminogen activator receptor 1; PAI-2 - plasminogen activator receptor 2; IHC – immunohistochemistry, RT-PCT - reverse transcription polymerase chain reaction; ISH – in-situ hybridisation; ELISA - enzyme-linked immunosorbent assay; GOJ –gastro-oesophageal junction.
*expression at invasive front

Chapter 3

Expression of cancer stem cell markers and uPAR in metastatic gastroesophageal cancer

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Contribution of authors:

DB – research proposal, concept development, patient identification and sample collection, data collection and analysis, interpretation of results, manuscript draft and revisions

LA, IA, MA, PC, NH – IHC staining and scoring

MA, WL, SP, AS – identification of patients, sample collection, manuscript revisions

TB, MC, KV, – interpretation of results, manuscript revisions

MR – concept development, interpretation of results, manuscript draft and revisions

Expression of Cancer Stem Cell Markers is Prognostic in Metastatic Gastroesophageal Adenocarcinoma.

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Abstract

Background: Gastroesophageal adenocarcinoma is a common and highly lethal malignancy. There is a growing evidence base to support the central role of cancer stem cells (CSCs) in the development and progression of metastatic disease. While numerous studies have shown the poor clinical outcomes associated with expression of the CSC markers CD44, CD133 and ALDH1 in locoregional gastroesophageal cancer, there is a paucity of data in distant metastatic disease. We aimed to investigate the prognostic significance associated with expression of CSC markers in metastatic gastroesophageal cancer.

Methods: We examined the immunohistochemical expression of CD44, CD133, and ALDH1 on metastatic deposits from gastroesophageal and gastric adenocarcinomas, and evaluated the association of CSC expression with clinicopathological factors, metastases biomarker urokinase plasminogen activator receptor (uPAR) expression, and overall survival (OS).

Results: Of the 36 patients included in the study, 16 (44%) were positive for CD44, 13 (36%) were positive for CD133, and 26 (72%) were positive for ALDH1. CD44 expression was significantly associated with poorer OS in univariate (HR 2.9 95%CI 1.3 – 6.9, p=0.008) and multivariate analyses (HR 2.5 95%CI 1.1 – 6.2, p=0.04). ALDH1 expression was significantly associated with poorer OS in univariate (HR 2.4 95% CI 1.01 – 5.7, p=0.04) analysis but was not significant in multivariate analysis. Both CD44 and ALDH1 expression were significantly associated with uPAR expression. We found no association between CD133 expression and OS.

Conclusions: CD44 expression on metastatic disease from gastroesophageal adenocarcinomas is an independent prognostic marker associated with poorer OS. These results expand current evidence to support the role of CSCs as biomarkers in metastatic gastroesophageal cancer.

3.1 Introduction

Gastroesophageal adenocarcinoma, a common and lethal malignancy, is a leading cause of cancer mortality worldwide[1]. Despite advances in treatment, prognosis remains poor due to high rates of recurrence after curative surgery, and limited response to systemic treatment in advanced disease[2]. There is an urgent need for novel therapeutic strategies to improve treatments for patients with gastroesophageal cancer.

The cancer stem cell (CSC) hypothesis seeks to explain the high rate of relapse and resistance to current anti-neoplastic treatments. The CSC model proposes that tumour formation, maintenance, and growth is driven by a small population of self-sustaining cells which possess stem cell properties of longevity and infinite proliferation, and are able to differentiate into the wide range of cells forming the heterogeneous tumour mass[3,4]. CSCs, first demonstrated in acute myeloid leukaemia, have been described in most solid tumours including breast cancer[5], prostate cancer[6], pancreatic cancer[7,8], melanoma[9], colon cancer[10,11], and brain cancer[12,13].

CSC theory has important clinical implications, as it infers that treatment should be directed to the small pool of CSCs, as well as the large, terminally differentiated tumour bulk. Lineage tracing studies show that CSCs are able to reconstitute the entire tumour bulk following chemotherapy[14]. Promising results from early clinical studies suggest that the inherent resistance of CSCs to chemotherapy and radiotherapy can be overcome by the combination of chemotherapy with CSC targeted treatment in gastric cancer[15,16].

While numerous proteins have been identified as potential markers of CSC in gastroesophageal cancer including CD24, CD49, Sox2, Oct4, and Nanog, the most consistent evidence is for three main markers; CD44, CD133 and ALD[17].

CD44 is a transmembrane glycoprotein with important roles in matrix adhesion, cell migration, growth, and survival[18,19]. CD44 positive cells from gastric cancer cell lines are shown to be more tumorigenic in mouse and *in vitro* models[20-22], and resistant to chemotherapy and radiotherapy[20,22]. CD44 is expressed in 44-63% of resected primary gastric cancers [23,24], and is associated with larger tumour size, depth of invasion, advanced TNM stage, and positive LN[24-26]. Primary tumour CD44 expression is an independent prognostic factor associated with increased risk of recurrence and poorer overall survival in resected gastric cancer[25-27].

CD133 is a cell surface transmembrane glycoprotein with a proposed role as an organiser of plasma

membrane topology[28]. Preclinical work shows that CD133 positive cells isolated from cell lines demonstrate stem cell properties, and are more resistant to chemotherapy[12,13,29]. Rates of CD133 expression on primary gastric cancer resection specimens range from 25 – 90%[30,31]. Expression on resected primary gastric tumours is associated with higher risk pathological features, and is an independent factor associated with worse clinical outcomes[24,30,32-35]

ALDH1 is a member of a family of intracellular enzymes contributing to cellular detoxification, differentiation, and drug resistance[36]. *In vitro,* ALDH1 positive cells from gastric cancer cell lines show self-renewal, heterogeneous linage and increased tumourogenicity[37]. Primary tumour ALDH1 expression is associated with higher TNM stage and pathological factors[24,38].

The CSC model proposes disseminated CSCs to be the source of metastases, either as primary circulating CSCs or by dedifferentiation though phenotypic plasticity[39]. The expression of CSC markers has been linked to the development of metastatic disease in a variety of malignancies including gastric[27,34], colorectal[40], breast[41] and pancreatic[7] cancer.

A key step in the formation of metastatic deposits is invasion of the tumour cells into the surrounding normal tissue. This is facilitated through the urokinase type plasminogen activator (uPA) system, the critical proteolytic pathway and predominate source of malignant plasminogen activation[42]. Expression of the uPA receptor uPAR is an important independent prognostic factor in many solid malignancies including gastroesophageal cancer[43-45], and has an emerging role in CSC signalling[46,47]. Although the expression of CSCs markers have been well characterised in resected locoregional gastroesophageal adenocarcinoma, there is no data on the expression of CSC markers in metastatic disease. In the current study, we aimed to analyse the IHC expression of CD44, CD133 and ALDH1 on metastatic gastric cancer deposits, and correlate expression with prior treatment, clinicopathological factors, uPAR expression, and clinical outcomes.

3.2 Material and Methods

3.2.1 Patient Population and Tissue samples

We retrospectively identified all patients with metastatic gastric or gastroesophageal junction adenocarcinomas treated at two Australian Tertiary Hospitals (Wollongong Hospital, St George Hospital) between 2010-2014 who had an available tissue sample from a metastatic site (n=50). 38 samples were suitable for staining and scoring. Clinicopathological variables extracted from patient records included: age, sex, tumour histological type, grade, site of metastases, Eastern Cooperative Oncology Group (ECOG) performance status, treatments, progression free survival (defined as the time from the date of primary treatment to the date of progression or death) and overall survival (time

from diagnosis to death from any cause). The study was approved by South Western Sydney Local Health District Human Research Ethics Committee (Project Number 15/072).

3.2.2 Immunohistochemistry

We used formalin fixed, paraffin embedded tissues from patients who underwent biopsy of a metastatic deposit from primary gastroesophageal adenocarcinoma. Immunohistochemical staining was done as previously reported [24]. Freshly cut 4 micron sections from patient tissue blocks were mounted on aminopropylethoxysilane precoated glass slides. Sections were deparaffinised in EZ Prep and washed in Reaction Buffer (Ventana Medical Systems, Arizona, US). The immunohistochemical staining was performed using defined protocols with the Ventana BenchMark Ultra Automated IHC/ISH slide staining system. Antigen retrieval was performed by incubation at 100°C at pH 9.0 for between 24 to 32 minutes. Incubation with primary antibodies was carried out at 37°C for one hour. Sections were incubated with the following antibody dilutions: anti-CD44 (clone EPR1013Y, Abcam) 1:200, CD133 (clone AC133, Miltenyi Biotec) 1:100, ALDH1 (clone 44, BD Transduction Laboratories) 1:100, and uPAR (clone R4, Dako) 1:100. A post primary endogenous peroxidase inhibition was performed by incubating the slides in 1% hydrogen peroxide for 15 min. Development of colour was achieved by 15 minutes incubation with diaminobenzidine solution, followed by counterstaining with haematoxylin. Sections from normal human epithelium, colon adenocarcinoma and normal human appendix were used as positive controls for CD44, CD133 and ALDH1 respectively [24,36]. All staining runs were accompanied by appropriate control slides.

3.2.3 Scoring of immunohistochemical staining

CSC scoring was performed by two independent pathologists blinded to clinical details (AL, and AI or NH). Previous reports have shown a significant correlation between CD44, CD133 and ALDH1 expression and prognosis in primary gastroesophageal cancer [17] . To remain consistent with the literature [24,27,48], CD44 and CD133 staining was considered positive if at least 10% of the tumour cells were stained. We noted a much higher proportion of ADLH1 positive cases than previous studies (only 5 negative cases using a 10% cutoff); accordingly we increased the threshold to 20% (positive result if 20% or more of the tumour cells stained). Staining in surrounding stroma was not included in the score for any CSC marker.

uPAR scoring was performed by a third blinded pathologist (MI) experienced with immunohistochemical analyses of the uPA system in cancer [43,49]. uPAR expression on cancer cells varies between the tumour core, and the invading edge of the tumour [49]. Analysis of uPAR expression was restricted to the peripheral invasion zone as this has been shown to be prognostic in gastric cancer [44]. Neutrophils were used as internal positive controls on each slide. Scoring was performed as

previously described; 0- No uPAR-positive cells; 1- Less than 1% uPAR-positive cells; 2- 1–5% uPAR-positive cells; 3- 5–10% uPAR-positive cells; 4- More than 10% uPAR-positive cells as previously reported for gastroesophageal cancer . Samples were considered to be uPAR positive if >5% of tumour cells were stained [43,44].

3.2.4 Statistical analysis

The primary endpoint of this analysis was overall survival (OS) by CSC expression. Summary statistics of patients' demographic and clinicopathological details, and staining status were provided in frequencies and percentages. Bivariate correlations between clinicopathological features and CD44, CD133, and ALDH1 expression were performed using the Fisher's exact test. A Cox proportional hazard model was used to estimate effects of CD44, CD133 and ALDH1 positivity on each survival endpoint; only covariates significant in univariate analysis were included in the multivariate model. uPAR expression was not included in the multivariate model as staining was only available for a subset of patients. All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC).

3.3 Results

3.3.1 Patient characteristics and correlation with CSC expression.

Characteristics of the 36 included patients are summarised in Table 1. Median follow-up was 5.2 months (Interquartile range 2.8 – 10.7 months). Consistent with the poor prognosis of this disease, most patients (n=32, 89%) had died of their disease. 17 (45%) of patients received treatment for loco-regional disease prior to developing metastases, although in all cases this was more than 6 months prior to biopsy. Most patients (n=32, 84%) received treatment for the metastatic gastroesophageal cancer including chemotherapy (usually a platinum, fluoropyrimidine and anthracycline combination), radiotherapy, or surgery (Table 1). Radiotherapy and surgery were employed as palliative local treatments for symptomatic metastases.

Table 1: Characteristics of included patients GOJ – gastroesophageal junction; * site of distal lymph nodes include mediastinal, supraclavicular, and para-aortic; **All prior treatment was curative intent

Characteristic	Number (%)
Age – median (range)	64 (39 – 78)
Sex, n (%)	
Male	29 (80)
Female	7 (19)
Primary tumour location	
GOJ	16 (44)
Gastric body	20 (56)
Site of metastatic biopsy	
Pulmonary	4 (11)
Peritoneum/omentum/ascites	18 (50)
Liver	7 (18)
Bone	2 (5)
Distal lymph node*	4 (11)
Soft tissue	2 (5)
ECOG performance status	
0 – 1	31 (86)
2 – 4	5 (14)
Prior Treatment**	
Surgery	13 (34)
Chemotherapy	13 (34)
Radiotherapy	5 (13)
Nil	21 (55)
Treatment for metastatic disease	
Chemotherapy	26 (68)
Immunotherapy	1 (3)
Radiotherapy	7 (18)
Surgery	3 (8)
Nil	6 (16)

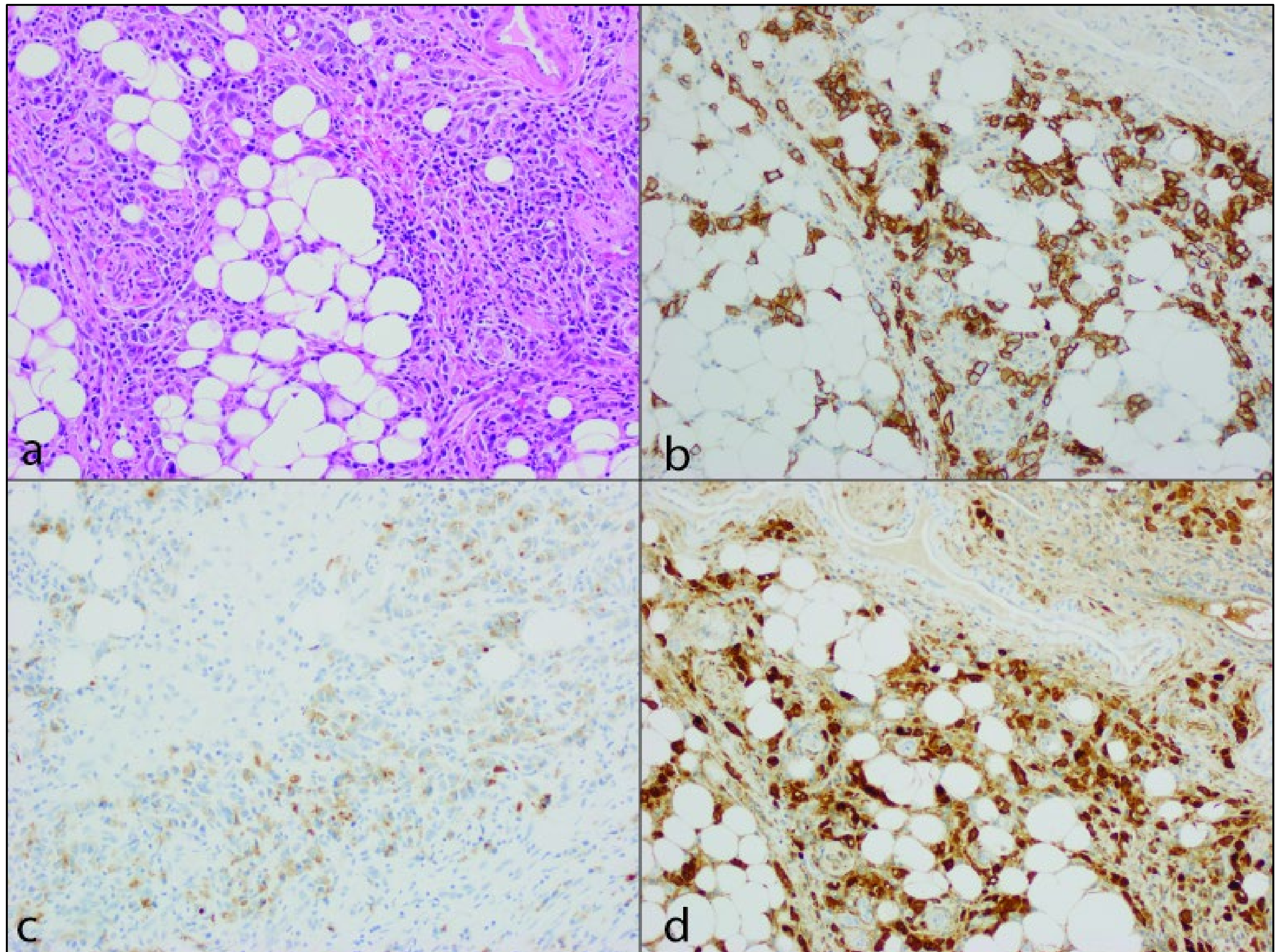

Figure 1: Representative staining of CSC markers on metastatic deposits with corresponding Hematoxylin and eosin stain (20x magnification). (a) Hematoxylin and eosin stain (b) membranous staining of CD44 (c) CD133 staining in apical membranes (d) cytoplasmic staining of ALDH1.

Of all cases, 16 (44%) were positive for CD44, 13 (36%) were positive for CD133, and 26 (72%) were positive for ALDH1. We found no association between CSC markers and clinicopathological features, including primary tumour location, site of metastatic disease or biopsy sample, previous chemotherapy exposure, or histopathology (Table 2).

Table 2: Association of CSC marker staining with clinicopathological features (n=36): GOJ – gastroesophageal junction;

	CD44		**CD133**		**ALDH1**	
Total (%)	16/36 (44%)		13/36 (36%)		26/36 (72%)	
% positive cells (mean)	10 – 100% (62)		10 – 100% (38)		10 – 100% (76)	
	Positive rate	P-value	Positive rate	P-value	Positive rate	P-value
Primary location						
GOJ	8/16	0.73	6/16	0.98	12/16	0.98
Gastric	8/20		7/20		14/20	
Sites of Metastatic disease						
Peritoneal/Omentum only	7/18	0.73	4/18	0.16	14/18	0.71
Other	9/18		9/18		12/18	
Previous Chemotherapy						
Yes	6/13	0.87	5/13	0.83	10/13	0.72
None	10/23		8/23		16/23	
Histopathology						
Well/mod differentiated	5/16	0.19	5/16	0.73	5/16	0.72
Poorly differentiated	11/20		8/20		15/20	

3.3.2 Correlation of CSC marker and uPAR expression

Samples including the peripheral invasion zone were available for 28 samples (8 samples excluded, due to insufficient tissue n=4, or the biopsy included tumour core only n=4). 9/28 (32%) samples were positive for cancer cell uPAR. CD44 and ALDH1 expression was significantly associated with tumour cell uPAR (p=0.02 and 0.03 respectively, Table 3), with higher tumour uPAR expression in CD44 and ALDH1 positive cases. There was no association between CD133 and uPAR expression.

Table 3: Association of CSC marker staining with uPAR staining (n=28). uPAR – urokinase plasminogen activation receptor

	Positive uPAR staining	**P-value**
CD44		
Positive	7/15 (54%)	0.02
Negative	2/13 (13%)	
CD133		
Positive	1/9 (11%)	0.10
Negative	8/19 (42%)	
ALDH1		
Positive	9/12 (43%)	0.03
Negative	0/7 (0%)	

3.3.3 CSC marker expression and prognosis

In univariate analysis, CD44 positive cases had a poorer OS than CD44 negative cases (HR 2.9 95%CI 1.3 – 6.9, p=0.008, Table 4). Similarly, ALDH1 positive cases had a poorer OS than ALDH1 negative cases (HR 2.4 95%CI 1.1 – 5.7, p=0.04). There was no significant difference in OS between CD133 positive and negative cases (HR 1.16 95% CI 0.57 – 2.4, p=0.67).

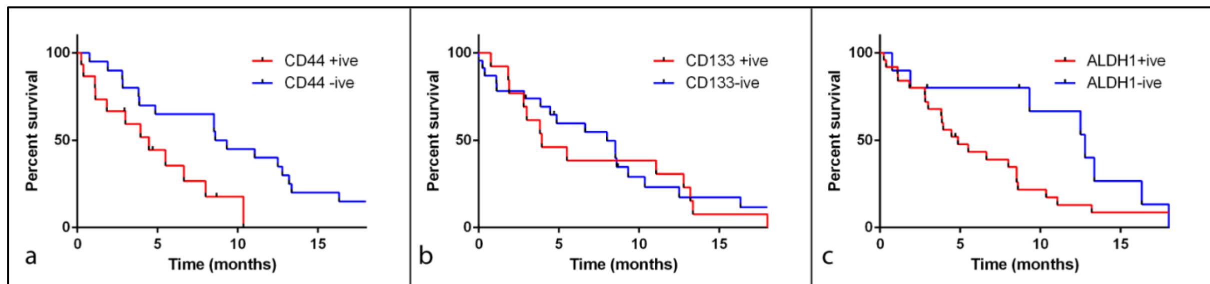

Figure 2: Kaplan-Meier survival curves for overall survival stratified by cancer stem cell marker expression (a) CD44: univariate HR 2.9 95%CI 1.3 – 6.9, p=0.008. (b) CD133: univariate HR 1.16 95% CI 0.57 – 2.4, p=0.67. (c)ALDH1: univariate HR 2.4 95%CI 1.1 – 5.7, p=0.04

In multivariate analysis, after adjusting for performance status, tumour grade, and treatment, CD44 positivity remained a significant independent predictor of OS (HR 2.5 95%CI 1.1 – 6.2, p=0.04), while ALDH1 became non-significant (HR 2.0 95%CI 0.86 – 5.1, p=0.1) (Table 4).

Expression of combinations of CSC markers was also assessed for association with OS. Patients with CD44+ive/ALDH1+ive expression (14/36, 39%) had a significantly poorer OS in univariate (HR 4.1 95%CI 1.7 – 9.5, p=0.0006) and multivariate analysis (HR 4.0 95%CI 1.6 – 10.1, p=0.002). No combination including CD133 was significantly associated with OS.

Table 4: Univariate Analysis and Multivariate analysis (significant values in italics) *ECOG – Eastern Cooperative Oncology group; HR – Hazard ratio; GOJ –gastroesophageal junction; CI – confidence interval;* * Radiotherapy and surgery were given as palliative local treatments only and therefore had no impact on survival, and were not incorporated into the multivariate model. Systemic treatment included chemotherapy or immunotherapy.

	Univariate		**Multivariate**	
Characteristic	**Hazard Ratio (95% CI)**	**P**	**Hazard Ratio (95% CI)**	**P**
ECOG performance status				
0-1	*1*	*0.0014*	1	0.75
2-4	*7.3 (2.1 – 25.0)*		1.2 (0.4 – 6.2)	
Age				
<65	1	0.79		
≥65	1.1 (0.5 – 2.2)			
Sex				
Female	1	0.42		
Male	1.5 (0.56 – 3.9)			
Primary location				
GOJ	1	0.09		
Gastric	0.55 (0.26 – 1.1)			
Site of Metastatic disease				
Peritoneal/Omentum only	1	0.16		
All other sites	0.60 (0.29 – 1.2)			
Histopathology				
Well/mod differentiated	*1*	*0.0003*	*1*	*0.007*
Poorly differentiated	*3.9 (1.8 – 8.6)*		*3.3 (1.4 – 7.9)*	
Treatment				
None	*1*	*0.001*	*1*	*0.03*
Systemic Treatment	*0.27 (0.12 – 0.62)*		*0.28 (0.1 – 0.88)*	
Radiotherapy	1.1 (0.5 – 2.9)	0.71*		
Surgery	2.2 (0.6 – 7.5)	0.19*		
CD44				
Negative	*1*	*0.008*	*1*	*0.04*
Positive	*2.9 (1.3 – 6.9)*		*2.5 (1.1 – 6.2)*	
CD133				
Negative	1	0.67		
Positive	1.16 (0.57 – 2.4)			
ALDH1				
Negative	*1*	*0.04*	1	0.1
Positive	*2.4 (1.01 – 5.7)*		2.0 (0.86 – 5.1)	

3.4 Discussion

In the CSC model, establishment and progression of metastatic disease is due to the dissemination of CSCs. While numerous previous studies have demonstrated expression of CSC markers in loco-regional gastroesophageal cancer to be significantly associated with clinical outcomes, the current study is the first to examine the expression of CSC markers in metastatic gastroesophageal cancer. We found expression of CD44 and ALDH1, but not CD133, on metastatic deposits to be significantly associated with poorer OS. In multivariate analysis, after adjusting for tumour grade, ECOG performance status, and treatment received, CD44 expression remained a significant prognostic factor associated with poorer OS (HR 2.5 95%CI 1.1 – 6.2, p=0.04). ALDH1 expression was not significantly associated with OS in multivariate analysis (HR 2.0, 95% CI 0.86 – 5.1, p=0.1), although the combination of CD44+/ALDH1+ive was strongly associated with poorer OS (HR 4.0 95%CI 1.6 – 10.1, p=0.002). This finding confirms previous work showing the importance of CSC expression, particularly CD44, as a biomarker in gastroesophageal cancer[24,27,34]. Our results did not show any association between CD133 with OS, either alone or in combination with CD44 or ALDH1. Recent work suggests that only a subset of CD133 positive CSCs are essential for tumour metastases[7]. We hypothesise that additional markers, such as CXCR4, are required in combination with CD133 to identify this key subgroup.

We also found expression of CD44 and ALDH1 to be significantly associated with expression of uPAR. Our results mirror previous work in other solid tumours showing co-expression of uPAR with CSC markers[50-52]. In addition to a well characterised role in the uPA system, there is increasing evidence suggesting uPAR has an important function in CSCs. uPAR overexpression is strongly correlated with the CSC properties of an invasive phenotype, drug resistance, and poor prognosis[53]. Moreover, signalling by uPAR induces stem cell like properties in breast, brain, lung and prostate cancer cells[46,47,54-56]. To the best of our knowledge, this study is the first to show the co-expression of CSC and uPAR in gastroesophageal cancers, and further supports the role of uPAR in CSCs.

In resected loco-regional gastroesophageal cancer, the proportion of CD44 and CD133 positive cases is estimated at 17-77% and 10-44% respectively[57]. Our results demonstrated a similar proportion of CD44 positive (44%) and CD133 positive (36%) cases, but a higher expression of ALHD1 than that seen in locoregional disease (73% positive cases compared to 50-55%)[24,38]. This is despite using a higher cut-off for positive cases (20% of cells stained compared to 10%). In addition, most ALDH1 cases were diffusely and strongly stained (mean proportion of positive cells 76%). Our results support a previously identified trend of higher ALDH1 expression on local nodal deposits. In a study comparing IHC expression of CSC between primary gastric cancers and matched lymph node metastases, Wakamatsu et al also found a higher expression of ALDH1, but not CD44 or CD133, in

the lymph nodes[24]. ALHD1 expression is strongly correlated with expression of matrix metalloproteases (MMPs), which are essential for extracellular matrix degradation and establishment of metastatic disease[38].

We were unable to show any significant association between CSC staining and other important clinicopathological factors. This is in contrast to other IHC studies which have shown strong associations between poor pathological factors, such as TNM stage, tumour invasion and grade with expression of CSCs[17]. The small sample size of our study is likely to be a contributing factor. It is interesting to note we did not find a higher CSC expression in patients with previous chemotherapy exposure. While CSCs are known to be relatively chemotherapy insensitive, leading to enrichment of CSCs with chemotherapy, modern CSC models describe a dynamic CSC population with a bidirectional pathway between CSC and differentiated cell populations[58]. As no patient had received chemotherapy within 6 months prior to the biopsy, it is likely that the CSC population had re-established equilibrium with the terminally differentiated tumour bulk.

The key role of CSCs in metastatic gastroesophageal cancer is supported by early clinical results of agents targeting the CSC pathway. For example, in a phase II study using the hedgehog inhibitor vismodegib with chemotherapy in metastatic gastric cancer, Yoon et al found a survival benefit restricted to patients who had a high expression of CD44[15]. Even more novel approaches using the combination of immunotherapy and CSCs are under investigation, with currently recruiting clinical trials employing immune targeting of CSC using dendritic cells[59]. The coexpression of CSC markers and uPAR may provide additional opportunities to target CSC using uPAR directed therapies[52].
It is important to acknowledge several limitations of this study. Firstly, we found that most gastroesophageal cancers, even when metastatic, have histological diagnosis on endoscopy and biopsy, rather than biopsy of metastatic deposits. This limited the available patient population and study size for the current work. Secondly, most samples used in the current study were biopsy specimens, rather than larger resection specimens, which did not allow exploration of tumour heterogeneity and differential expression of CSC makers. Thirdly, due to technical limitations, uPAR staining was available for most, but not all patients, limiting incorporation into the multivariate OS analysis.

In conclusion, expression of the CSC marker CD44 is an independent prognostic factor associated with poorer OS in metastatic gastroesophageal cancer. This study provides further evidence that expression of CSC markers a valid biomarker in gastroesophageal cancer, and highlights importance of CSCs in all stages of gastroesophageal cancer.

3.5 Appendix: uPAR expression in primary and metastatic gastroesophageal adenocarcinoma (GOC).

3.5.1 Background

Expression of uPAR is a clinically relevant biomarker in resected gastroesophageal cancer (Chapter 2[45]). High expression of uPAR in primary tumours is associated with high risk clinicopathological features and poorer OS. Several studies have shown a strong association of uPAR staining in primary tumours and the presence of lymph node and distant metastases in gastroesophageal cancer. As demonstrated in Chapter 2 (Section 2.3.5) uPAR expression in the primary tumour was significantly associated with nodal metastases (OR 2.5, $p<0.0001$), liver metastases (OR 2.5, $p=0.01$), and peritoneal metastases (OR 3.2, $p<0.0001$).

There is however, much more limited evidence regarding uPAR expression on distant disease. One study by Hong *et al* compared uPAR expression in primary tumours and matched regional lymph nodes from 9 patients with gastric cancer, and found similar expression of uPAR in the primary (56%) and lymph nodes (67%) [60]. There are no studies directly comparing primary tumour uPAR expression with distant metastases in gastroesophageal cancer. Using matched primary tumours and liver metastases from 14 colorectal cancer patients, Illemann *et al* found only a minority of liver metastases demonstrated a similar uPAR expression to the primary tumour, with most metastases exhibiting little uPAR expression [61]. In addition, as discussed in Chapter 1 (section 1.2.4.4), uPAR expression on disseminated tumour cells (defined by CK+ phenotype) in the bone marrow following curative treatment in locoregional disease, was significantly associated with subsequent disease relapse and poorer clinical outcomes [62].

The uPA system is expressed on both cancer cells and the supporting stroma [42]. uPAR is known to be expressed by many cell types within the tumour, including cancer cells, macrophages, myofibroblasts, neutrophils, and nerves. It is postulated that the expression of uPA and uPAR on stromal and tumour cells, respectively, at the invasive front of a tumour, facilitate proteolysis required for invasion [63].

3.5.2 Aims

1) Compare uPAR expression between primary tumours and metastatic deposits in GOC
2) Determine the association of uPAR expression on GOC and overall survival using:
    - Expression at the tumour core or invading edge of tumour
    - Stromal (macrophages and myofibroblasts) and cancer cells uPAR expression

3.5.3 Methods

uPAR expression of primary (n=12) and metastatic (n=33) tumour was analysed using IHC (methods as per chapter 4). uPAR scoring was performed individually on cancer cells, macrophages, and myofibroblasts. The proportion of uPAR positive cells was scored by evaluation of the whole slide. Separate values were obtained for each cell type with the following scores: uPAR-score 0: No uPAR-positive cells; uPAR-score 1: Less than 1% uPAR-positive cells; uPAR-score 2: 1‐5% uPAR-positive cells; uPAR-score 3: 5‐10% uPAR-positive cells; uPAR-score 4: More than 10% uPAR-positive cells. Neutrophil positive uPAR staining on each slide was used as an internal control.
In addition, all three cell types were scored separately for the invasion front at the tumour periphery, and in the tumour core. As a consequence of limited tissue available in biopsy specimens, tumour core scores were only available for 21 metastatic samples (63%), as most biopsy specimens included only the invasion front of the tumour.

The association of uPAR expression and OS was determined for each cell type, at both the invasion front and the tumour core. Given the small sample size and the absence of a standardised cut-off for uPAR expression, a range of values for uPAR expression was tested on each cell type. The cut-off with strongest association is presented.

3.5.4 Results

uPAR expression was evaluable on 45 samples in total (43 invasive front, 24 tumour core, 21 both). Characteristics of included patients are shown in Table 1. Representative images of tumour and stromal cell uPAR staining are shown in table A1.

Table A1: Characteristics of included patients. Treatment subgroup total is greater than 45 as individual patients may have received more than one treatment modality.

Characteristic	**Number (%)**
Age – median (range)	65 (40 – 82)
Age < 65 yrs	21 (44)
Age ≥ 65 yrs	24 (56)
Sex, n (%)	
Male	37 (82)
Female	8 (18)
Primary tumour location	
GOJ	17 (38)
Gastric body	28 (62)
Stage	
II	8 (18)
III	3 (7)
IV	34 (75)
Site of biopsy	
Primary tumour	12 (27)
Metastasis	33 (73)
Tumour Grade	
Well/Mod diff	23 (51)
Poorly diff	22 (49)
ECOG performance status	
0 – 1	39 (87)
2 – 4	6 (13)
Treatment	
Chemotherapy	29 (67)
Radiotherapy	7 (16)
Surgery	14 (33)
Nil	8 (19)

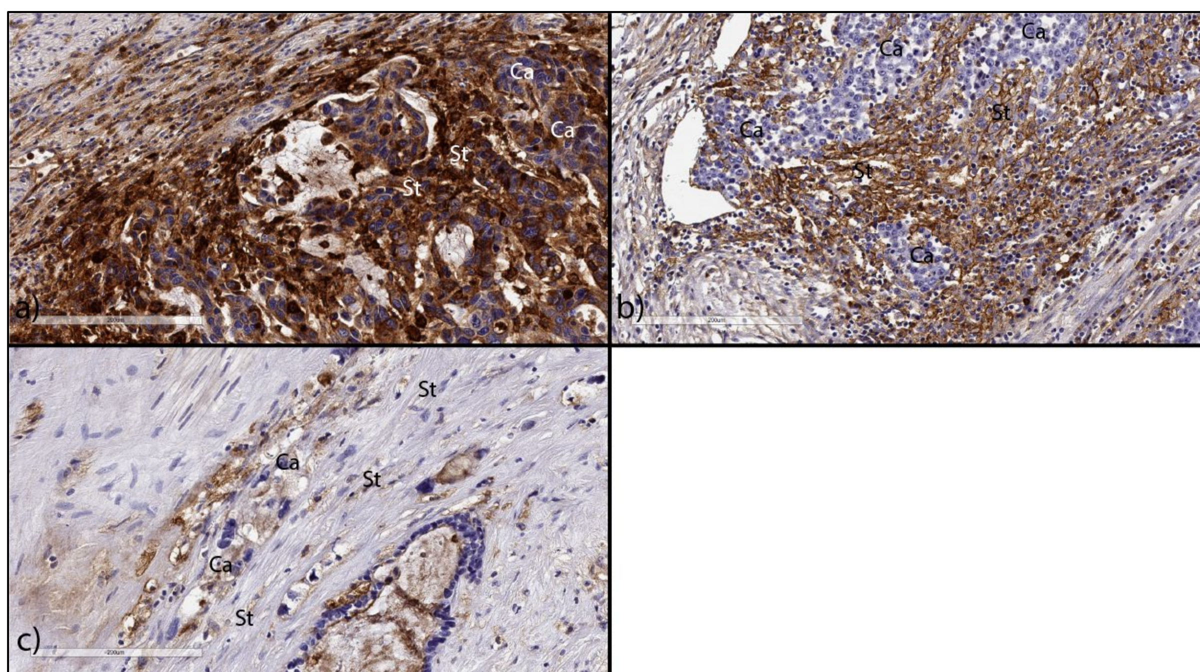

Figure A1: Three patterns of uPAR staining. a) There is widespread strong uPAR expression on cancer cells (Ca) and macrophages and myofibroblasts (stromal cells, St). b) Strong stromal but weak cancer cell staining. c) weak stroma staining with strong cancer cell

uPAR scores are summarised below (Table A2). There was non-significant trend to higher uPAR expression on cancer cells in the invasion front (Chi Sq p= 0.069). There was no difference in macrophage or myofibroblasts expression between tumour areas.

Table A2: uPAR expression score for Tumour Core and Invasion Front. Results are presented as absolute values and percentages for each subgroup to facilitate comparison.

			uPAR score		
Cell Type	0 (%)	1 (%)	2 (%)	3 (%)	4 (%)
Tumour Core (n=24)					
Cancer cell	8 (33)	9 (38)	1 (4)	3 (13)	3 (13)
Macrophage	2 (8)	3 (13)	7 (29)	1 (4)	11 (46)
Myofibroblast	7 (29)	4 (17)	4 (17)	4 (17)	5 (21)
Invasion Front (n=43)					
Cancer cell	11 (26)	6 (14)	9 (21)	5 (12)	12 (28)
Macrophage	3 (7)	4 (9)	10 (23)	8 (19)	18 (19)
Myofibroblast	15 (35)	10 (23)	8 (19)	2 (5)	7 (16)

3.5.4.1 uPAR expression at the tumour invasive Edge (n=43)

There was no significant difference in uPAR expression on tumour cells (mean uPAR score primary tumours 1.8 vs 2.1, t-test p=0.62), myofibroblasts (mean 2.0 vs 1.2, p=0.1), or macrophages (mean 3.3 vs 2.6, p=0.08) (Figure A2) between primary and metastatic sample. Similarly, there was no significant association between tumour cell uPAR score and clinicopathological variables such as primary tumour location (Fishers exact p=0.08) or tumour grade (p=0.24).

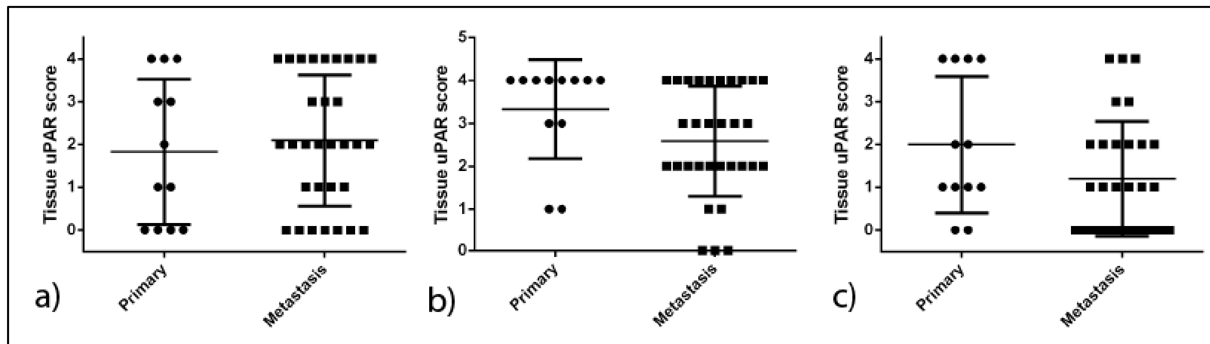

Figure A2: Comparison of uPAR score between primary and metastatic samples. There was no significant difference in uPAR score on a) tumour cells (p=0.62) b) macrophages (p=0.08) or c) myofibroblasts (p=0.1)

In univariate analysis, uPAR expression on tumour cells was significantly associated with poorer OS (uPAR score 2-4 compared to 0-1, HR 2.5 95% CI 1.1 – 5.6, p=0.02) (Figure A3, Table A3). In contrast, there was no association with uPAR expression on macrophages (uPAR score 0-2 versus 3 - 4, p=0.9) or myofibroblasts (uPAR score 0-1 versus 2 -4, p=0.4), also it is interesting to note a trend to improved survival with higher uPAR score on myofibroblasts, the opposite pattern seen on tumour cells (Figure A2). As expected, other important prognostic factors including ECOG performance status, primary tumour location, stage, histopathological grade, and receipt of treatment was significantly associated with OS (Table A3).

The association of uPAR expression on tumour cells remained significant in multivariate analysis (HR 1.5 95% CI 1.1 – 2.1, p=0.0004) confirming uPAR expression as an independent prognostic factor (Table 3).

When analysis is restricted to metastatic patients only (n=31), high uPAR score remains significantly associated with poor OS in univariate (HR 2.5 95%CI 1.1 – 5.7, p=0.03) and multivariate analysis (HR 1.5 95% CI 1.1 – 2.1, p<0.0001).

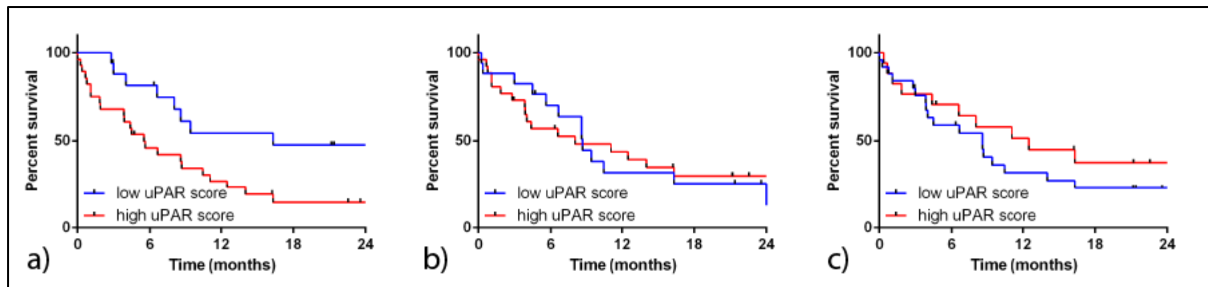

Figure A3: Association of uPAR score with OS (n=43). Patients with a high uPAR score on a) tumour cells have a significantly worse OS (p=0.02) but not b) macrophages (p=0.9) or c) myofibroblasts (p=0.4)

Table A3: Univariate and multivariate analyses for invasion edge uPAR scores and OS. Cancer cell uPAR expression was the only cell type with a significant univariate association with OS and therefore the only uPAR score included in the multivariate model.

	Univariate		**Multivariate**	
Characteristic	**Hazard Ratio (95% CI)**	**P**	**Hazard Ratio (95% CI)**	**P**
ECOG performance status				
0-1	*1*	*<0.0001*	1	0.9
2-4	*11.2 (3.2 – 39.9)*		1.1 (0.2 – 5.8)	
Age				
<65	1	0.47		
≥65	0.77 (0.4 – 1.6)			
Sex				
Female	1	0.8		
Male	1.1 (0.4 – 3.0)			
Primary location				
GOJ	1	0.02	*1*	*0.02*
Gastric	0.43 (0.20 – 0.90)		*0.4 (0.1 – 0.9)*	
Stage				
II-III	*1*	*0.0002*	*1*	*0.001*
IV	*9.4 (2.2 – 40)*		*6.3 (2.0 – 20.0)*	
Histopathology				
Well/mod differentiated	*1*	*<0.0001*	1	0.3
Poorly differentiated	*4.3 (2.0 – 9.4)*		1.7 (0.6 – 4.8)	
Treatment				
None	*1*	*0.004*	*1*	*0.0004*
Yes	*0.23 (0.1 – 0.6)*		*0.5 (0.1 - .6)*	
Cancer cell uPAR expression				
0-1	*1*	*0.02*	*1*	*0.0004*
2-4	*2.5 (1.1 – 5.6)*		*1.5 (1.1 – 2.1)*	
Macrophage uPAR expression				
0-2	1	0.9		
3-4	1.0 (0.5 – 2.1)			
Myofibroblast uPAR expression				
0-1	1	0.4		
2-4	0.75 (0.4 – 1.5)			

3.5.4.2 uPAR expression at the Tumour Core (n=24)

While there was no difference between uPAR expression on tumour cells between primary tumours and metastatic deposits (p=0.8), we found a lower uPAR expression on macrophages (p=0.01) and myofibroblasts (p=0.006) on metastatic sample (Figure A4).

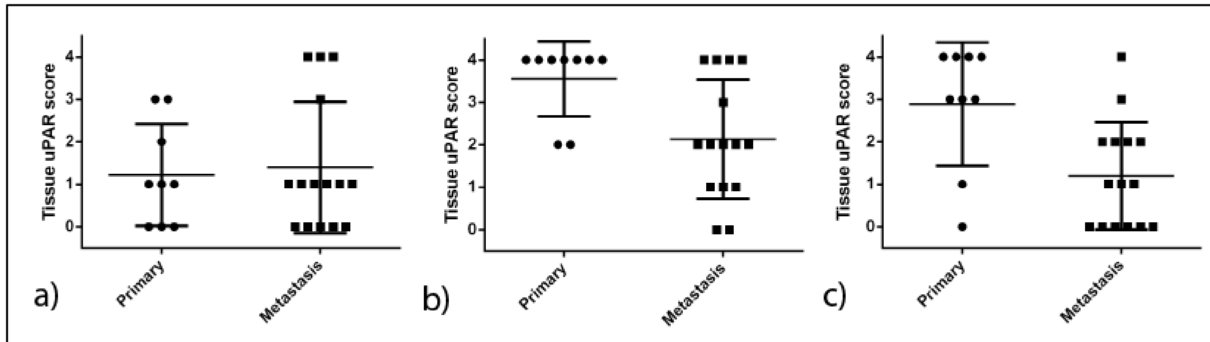

Figure A4: Comparison of uPAR score between primary and metastatic samples (n=24). There was no significant difference in uPAR score on a) tumour cells (t-test p=0.77) but significantly lower uPAR scores on b) macrophages (mean uPAR score 3.5 vs 2.1, p=0.01) or c) myofibroblasts (mean uPAR score 2.9 vs 1.2p=0.006)

Although limited by sample size, we did not find a significant association of tumour core cancer cell (uPAR score 0-1 vs 2-4, p=0.2) or myofibroblasts (uPAR score 0-1 vs 2-4, p=0.9) uPAR score and OS (figure A5). Patients with a higher uPAR score on macrophages had an improved OS (uPAR score 0-2 vs 3-4, HR 0.3 95% CI 0.1 – 0.7, p= 0.01). In view of the small sample size, no multivariate analyses were undertaken.

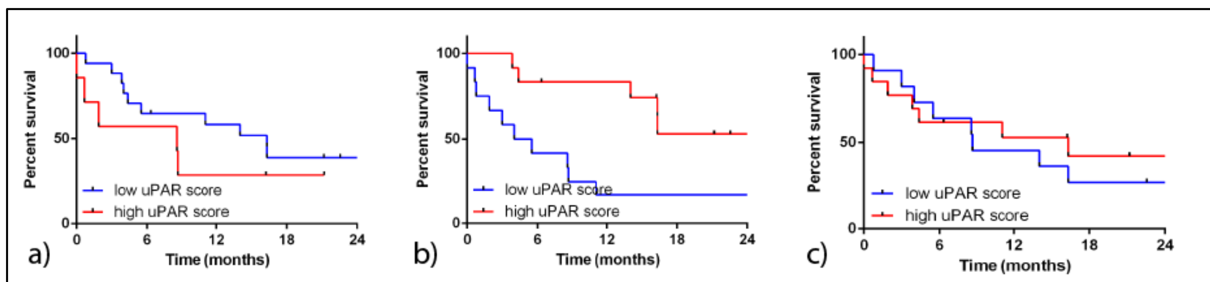

Figure A5: Association of tumour core uPAR score and OS (n=24). While there was no significant association between OS seen for a) cancer cell (p=0.2) or c) myofibroblasts (p=0.9), b) high macrophage uPAR expression was significantly associated with improved OS (p=0.01)

3.5.5 Discussion

Our results confirmed that uPAR expression on cancer cells at the invasive edge of tumours is an independent prognostic factor in gastroesophageal cancer. After controlling for stage, primary tumour location, ECOG performance status, and histopathological grade, cancer cell uPAR expression was associated with poorer OS (HR 1.5, p=0.0004). This is consistent with previous studies as discussed in chapter 2.

Moreover, we show that cancer cell uPAR expression at the invasive edge of tumours remains a significant independent prognostic factor when analysis is restricted to metastatic deposits of gastroesophageal cancers (HR 1.5, p<0.0001). uPAR expression on metastatic gastroesophageal cancer has not been previously reported.

Consistent with the results of Hong *et al* [60], we found similar uPAR scores between primary and metastatic tumour samples at the invading edge. Our results are in contrast to Illemann et al who found all primary tumours, but only a minority (5/14 patients, 36%) of liver metastases, demonstrated strong uPAR expression on tumour and stromal compartments on the invasive edge of the tumour[61]. It is important to note however, that this study was conducted on liver metastases from colorectal cancer, with most metastases exhibiting a pushing, rather than an invading, growth pattern. Interestingly, we found contrasting results from the stromal uPAR staining in the tumour core. Not only was there significantly lower expression of uPAR on stromal cells in the tumour core on metastatic samples compared to primary tumours, higher macrophage uPAR score in the tumour core was associated with an improved OS in univariate analyses (HR 0.3. p=0.01). Two previous studies examining the prognostic association of uPAR expression on stromal cells within the tumour cores of primary gastroesophageal cancers did not find significant prognostic associations[43,64].

While provocative, there are three important caveats to these results. Firstly the metastatic samples were taken from different patients to the primary samples, which introduce confounders into these results. Ideally this study should be repeated using matched metastatic and primary tumour samples from the same patient. Secondly, the uPAR score is presented as a proportion of cells expressing uPAR, rather than an absolute number. Metastatic samples may have much higher numbers of macrophages and myofibroblasts in the samples, leading to a lower proportion of uPAR positive cells in primary tumours. We plan to re-stain these samples with CD68, a macrophage marker, to provide clarity on this issue. Finally, it is important to acknowledge the limited numbers in this study which limits definitive conclusion.

3.5.6 Conclusion

uPAR expression on cancer cells at the invasive edge of metastatic tumours is an independent prognostic factor in gastroesophageal cancer. We found provocative results regarding uPAR stromal staining, particularly the improved survival associated with high macrophage uPAR expression in the tumour core, which require further validation due to limitations addressed above.

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Chapter 4

Circulating Tumour Cell Enumeration in gastroesophageal cancer

Brungs D, Lynch D, Luk AW, Minaei E, Ranson M, Aghmesheh M, Vine KL, Carolan M, Jaber M, de Souza P, Becker TM. Cryopreservation for delayed circulating tumor cell isolation is a valid strategy for prognostic association of circulating tumor cells in gastroesophageal cancer. World journal of gastroenterology. 2018 Feb 21;24 (7):810 (See appendix 1)

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DL, AL – protocol development, sample processing, manuscript revisions

EM - sample processing, manuscript revisions

MJ - patient identification and recruitment,

MA - patient identification and recruitment, interpretation of results, manuscript revisions

MC, KV, PdS,– interpretation of results, manuscript revisions

MR, TB– concept and protocol development, interpretation of results, manuscript draft and revisions

Cryopreservation for delayed circulating tumor cell (CTC) isolation is a valid strategy for prognostic association of CTCs in gastroesophageal cancer

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Abstract

Background/Aim: Circulating tumor cells (CTCs) are an important circulating biomarker in gastroesophageal cancer. However current techniques for CTC isolation require prompt processing of prospectively collected blood samples at specialised research facilities limiting widespread application. This study aimed to demonstrate the feasibility of cryopreservation of peripheral blood mononuclear cells (PBMCs) for prognostic CTCs detection in gastroesophageal cancer.

Method: 7.5 ml blood samples were collected in EDTA tubes from patients with gastroesopheagal adenocarcinoma. CTCs were isolated by EpCAM based immunomagnetic capture using the IsoFlux platform. Paired specimens from the same blood draw were used to compare CTC isolation from fresh and cryopreserved peripheral blood mononuclear cells (PBMCs). CTCs isolated from pre-treatment cryopreserved PBMCs were examined for associations with clinicopathological variables and survival outcomes.

Results: While there was a significant trend to a decrease in CTC numbers associated with cryopreserved specimens (mean number of CTCs 34.4 vs 51.5, p=0.04), this was predominately in samples with a total CTC count of >50, with low CTC count samples less affected (p=0.06). Duration of cryopreservation did not affect number of CTCs. CTCs were isolated in most patients (95.5%), with higher CTC counts correlated with metastatic disease, and a CTC count >17 significantly associated with a poorer overall survival in multivariate analysis (HR 3.7 95%CI 1.2 – 12.4, p=0.03).

Conclusion: We describe a feasible protocol for PBMC cryopreservation for delayed CTC isolation to assist with sample collection, transporting and processing. A high number of CTCs in cryopreserved specimens remained a poor prognostic factor in our validation cohort.

4.1 Introduction

Circulating tumour cell (CTC) analysis continues to be a rapidly developing field in oncology, offering a promising tool to both prognosticate and guide managements for patients[1]. Despite recent advancements in the field, one persisting challenge to the widespread adoption of CTC analysis for translational clinical trials or routine clinical care is the limited time frame considered best for blood processing and CTC isolation. Usually fresh blood is processed for CTCs within 24 hours after blood draw, requiring prompt transfer to specialised centres for CTC isolation and analysis, which offers significant logistical challenges[2]. To overcome this issue, some studies use blood collection tubes that contain fixatives. Fixation of blood samples can allow CTC processing delayed by several days which has proven very useful for some CTC analyses[3,4]. However, fixatives may interfere with down-stream molecular analyses that require isolation of nucleic acids[5].

An alternative is the use of cryopreservation protocols for peripheral blood nuclear cells (PBMCs) to allow delayed CTC isolation from these cells followed by CTC analysis. Cryopreservation should overcome fixation related analysis limitations and allow a far more flexible time frames for batched CTC processing. However, a defined, robust protocol that is proven to enable analysis of the same or at least a relevant proportion of CTCs to that found in fresh samples, needs to be adopted and confirmation is needed whether cryopreserved CTCs can still predict disease outcome.

The advantage of cryopreservation of PBMCs is that it requires only minimal local processing, possible in most diagnostic settings, as well as feasible cryostorage and frozen transport of PBMC samples.

While there are a large number of approaches used to isolate and identify circulating tumor cells (recently reviewed by van der Toom et al[6]) , the best established and widely used is with the CellSearch™ system (Menarini-Silicon Biosystems), which uses positive immunomagnetic isolation of epithelial cell adhesion molecule (EpCAM, an epithelial cell marker) expressing cells followed by cytokeratin (CK), CD45, and DAPI staining[2]. The CTCs are then identified with automated immunofluorescence microscopy, defined by an EpCAM/CK/DAPI positive and CD45 negative phenotype. CellSearch CTC counts have shown to be prognostic in large patient series in a variety of cancers[7-9], including gastroesophageal cancer[10-12], but the instrument offers limited sensitivity in resectable gastroesophageal cancer, with CTCs detected in less than 15% of patients[10,13].

The IsoFlux system (Fluxion) uses a similar definition of CTCs to CellSearch (EpCAM/CK/DAPI positive, CD45 negative phenotype), but has shown a greater sensitivity for CTC detection[14-16]. This platform uses EpCAM targeted immunomagnetic isolation of CTCs within a microfluidic setting,

improving isolation of CTCs with lower EpCAM expression, minimising leukocyte contamination, and allowing downstream applications including staining, enumeration, or sequencing as shown for fresh blood samples [16].

Here, we use a viable method of PBMC cryopreservation that allows subsequent isolation and immunocytochemical analysis of CTCs. We demonstrate the feasibility of PBMC cryopreservation for delayed CTC isolation using paired cryopreserved and freshly processed blood samples drawn at the same time from patients with gastroesophageal adenocarcinoma. Importantly, we also provide data confirming that cryopreserved CTCs remain clinically applicable as a circulating prognostic marker for overall survival.

4.2 Methods

4.2.1 Patient Population

Blood samples were collected from patients with histologically confirmed distal oesophageal, gastroesophageal junction, or gastric adenocarcinomas treated at Wollongong Hospital, Australia. Blood samples were collected in 7.5 ml EDTA Vacutainer tubes (Sarstedt AG & Co.) and maintained at room temperature until processing.

In the initial cohort (Cohort 1) to confirm the feasibility of cryopreservation, 15 patients with gastroesophageal carcinomas had 2 specimens taken during the one blood draw, one processed within 24 h (“fresh” specimen), and one cryopreserved with delayed CTC isolation and analysis (“cryopreserved” specimen). Pre-treatment blood samples were cryopreserved from a second, larger cohort of patients for correlation with clinical outcomes (Cohort 2). The study was approved by South Western Sydney Local Health District Human Research Ethics Committee (Project Number 15/072). A written informed consent was obtained from each participant before sample collection.

4.2.2 Sample Preparation

Blood samples were processed within 24 h to recover the PBMC fraction using 50 ml SepMate tubes and Lymphoprep according to manufacturer’s instructions (Stemcell Technologies, Vancouver, BC, Canada).

PBMCs used for fresh analysis were resuspended in Isoflux Binding Buffer and immediately processed for CTC isolation (see below). PBMCs for cryopreservation were well resuspended in 1 ml of diluted plasma (the supernatant of the PBMC preparation from the matching patient) with the

addition of 7.5% final DMSO, and stored at -80°C until further processing. Cryopreserved samples were thawed according to the protocol from Fluxion Biosciences, San Francisco, California, USA [17]. In brief, warmed (37°C) thawing buffer, consisting of RPMI 1640 with 10% Fetal Bovine Serum (FBS, Bovogen Biologicals, Australia) and 50 Unit/ml Benzonase (Sigma-Aldrich, Germany), was added to thawed samples, washed once in thawing buffer, and resuspended in IsoFlux Binding Buffer with 5% FBS.

4.2.3 Circulating Tumor Cell Isolation, staining, and imaging

As per Fluxion protocol, immunomagnetic beads preconjugated with anti-EpCAM antibodies (CTC Enrichment Kit; Fluxion Biosciences Inc) were added to PBMCs suspended in IsoFlux Binding Buffer, and incubated for 90 min at 4°C with passive mixing on a rotator. Samples were then loaded into the sample well of the microfluidic cartridge and underwent immunomagnetic isolation of CTCs with the IsoFlux using the standard protocol (Fluxion Biosciences Inc).

Recovered CTCs were blocked with a final concentration of 1.2 µg/µl mouse IgG in binding buffer (Jackson ImmunoResearch, Baltimore, PA) for 30 min, washed and fixed in fixing solution (Fluxion Biosciences Inc). The CTCs were then blocked in 10% FBS in binding buffer for 15 min, then underwent immunofluorence staining for anti-CD45 antibody conjugated to Alexa Fluor 647 (Biolegend, Clone HI30). The CTCs were also stained for urokinase plasminogen activator receptor (uPAR, CD87), a key receptor in the plasminogen activator system and clinically relevant biomarker in primary gastroesophageal cancer [18] (see also Chapter 5 Appendix), using anti-uPAR antibody conjugated to AF594 (ThermoFischer, Clone R4). After permeabilization with 0.1% Triton X-100, cells were probed with anti-cytokeratin antibody conjugated to FITC (Sigma-Aldrich, Clone PCK-26). CTCs were finally stained with Hoechst and mounted using Isoflux mounting media to 24-well glass bottom plates (MoBioTec, Goettingen, Germany) for imaging.

Imaging was performed with an inverted epifluorence microscope (Leica DMi8, Leica Microsystems Pty Ltd) using the Leica Application Suite. Cells were considered CTCs if they were CK positive, CD45 negative, nucleated and morphologically intact. The proportion of uPAR positive CTCs was recorded.

4.2.4 Statistical Analysis

The CTC recovery from matched cryopreserved and fresh samples were compared with the paired t-test. Correlation between cryopreservation time and CTC number was described with a Pearson correlation coefficient, and the Fisher exact test and t-test were used to compare the status of CTCs with categorical clinicopathologic factors.

For survival analyses, in the absence of established cut-offs for prognostic CTC numbers, the median CTC count (17) was used as the discriminator between high and low CTC counts. Survival analyses are conducted using Kaplan-Meier methods, with median survival reported. Unadjusted and multivariable Cox proportional hazards regression analyses were used to estimate the association between CTC counts and survival, and to calculate corresponding hazard ratios (HRs) and 95% confidence intervals (CIs). The following variables were included in the multivariate model: age, sex, ECOG, TNM stage, primary tumor location, and CTC count. All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC).

4.3 Results

4.3.1 Matched fresh and cryopreserved specimens (Cohort 1)

Matching parallel blood samples, collected from 15 gastroesophageal cancer patients (10 patients had blood taken prior to treatment, 5 patients were already on treatment), that had either been cryopreserved before CTC processing or were processed fresh, were compared. Cryopreservation of PMBCs lasted from 2 weeks to 25.2 months (median 14.6 months). There was no significant correlation between cryopreservation time and CTC number (Pearson r -0.25, p=0.09). CTCs isolated from cryopreserved samples appeared morphologically similar to fresh samples (Figure 1). There was an overall trend for smaller detectable CTC numbers isolated from the cryopreserved samples compared to fresh samples that reached significance (mean number of CTCs 34.4 cryopreserved vs 51.5 fresh, p=0.04, Figure 2), however this difference was predominately attributable to a larger fall in CTC numbers in samples with very high CTC counts (>50 CTCs in the fresh specimen). There was no significant difference in CTC count between cryopreserved and fresh samples for specimens with CTC count less than 50 (n=11 patients, mean number of CTCs 10.7 vs 16.3, p=0.06). Thus CTC loss by cryopreservation in patient samples with low CTC counts appears relatively minor (mean proportion of CTCs lost in cryopreserved samples = 23.95%).

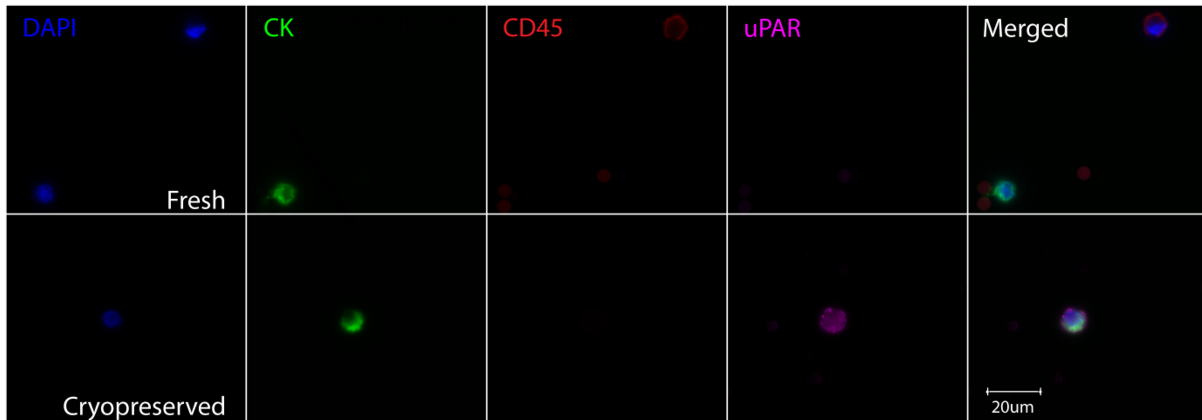


Figure 1: **Representative images of CTC isolation from fresh and cryopreserved samples demonstrating preservation of leukocyte and CTC morphology**. The fresh sample demonstrates a nucleated CK+/CD45- CTC which is uPAR negative, as well as a CK-/CD45+ leukocyte. The cryopreserved sample shows a uPAR positive CTC.

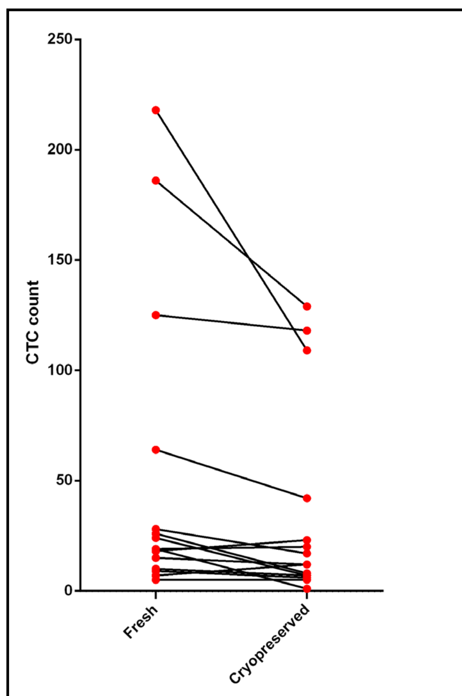


Figure 2: CTC enumeration by processing method. Mean number of CTCs isolated in the fresh specimens were higher than in the matched cryopreserved sample (mean difference in CTCs 17.1 95%CI 0.7 – 33.6, p=0.043). This difference was mostly driven by larger falls in CTC counts in samples with high numbers of CTCs (>50 CTCs in fresh samples), with no significant difference in CTC counts for samples with less than 50 CTC in the fresh specimen (p=0.06).

4.3.2 Cryopreserved CTCs and clinical outcomes (Cohort 2)

A larger cohort of 43 gastroesophageal cancer patients (Cohort 2) was analyzed to validate whether detectable CTC counts post cryopreservation correlated to disease outcomes. All patient samples were taken prior to treatment commencement and had undergone cryopreservation before CTC isolation. Cohort 2 included the 10 treatment naive patients from Cohort 1. Patient characteristics of Cohort 2 are summarised in Table 1. 24 patients had resectable disease (Stage II or III). Post CTC evaluation, 11 of these patients received neoadjuvant chemoradiotherapy prior to resection (CROSS regimen[19] – weekly carboplatin AUC2 and paclitaxel 50mg/m^2 with concurrent radiotherapy), 3 received perioperative chemotherapy (MAGIC regimen[20] – three preoperative and three postoperative cycles of epirubicin 50mg/m^2 and cisplatin 60mg/m^2 on day 1, and continuous fluorouracil infusion 200mg/m^2/day for 21 days), and 10 had surgery alone. 19 patients had metastatic disease (stage IV). Most of these patients received chemotherapy (7 patients: platinum and capecitabine doublet, 3 patients: anthracycline, capecitabine, and platinum triplet, 1 patient: irinotecan or paclitaxel monotherapy), immunotherapy (2 patients), and 6 patients received no active systemic treatments.

			CTC count	
		All Patients [%] n=43	**Low [CTC≤17] n=23**	**High [CTC >17] n=20**
Age				
	Mean (range)	64 (39 – 89)	65 (39 – 89)	64 (48 – 83)
Sex				
	Male	32 (74.4 %)	15 (65.2 %)	20 (85.0 %)
	Female	11 (25.6 %)	8 (34.8 %)	3 (15.0 %)
ECOG				
	0-1	36 (83.7 %)	22 (95.6 %)	14 (70.0 %)
	2-4	7 (16.3 %)	1 (4.3 %)	6 (30.0 %)
Primary Tumor Location				
	Distal Oesophageal	12 (27.9 %)	8 (34.8 %)	4 (20.0 %)
	Gastroesophageal junction	14 (32.6 %)	4 (17.4 %)	10 (50.0 %)
	Gastric	17 (37.5 %)	11 (47.8 %)	6 (30.0 %)
Stage				
	II	18 (41.9 %)	13 (56.5 %)	5 (25.0 %)
	III	6 (14.0 %)	4 (17.4 %)	2 (10.0 %)
	IV	19 (44.2 %)	6 (26.1 %)	13 (65.0 %)

Table 1: Characteristics of patients in Cohort 2. CTC – circulating tumor cell; ECOG – Eastern Cooperative Oncology Group performance status

CTCs were detected in 42/43 patients (95.5%), with a median CTC of 17 (interquartile range 8 – 38). Patients with metastatic disease had a higher number of CTCs than those with resectable disease (Figure 3, mean CTC count 53.8 vs 15.8, p=0.0013).

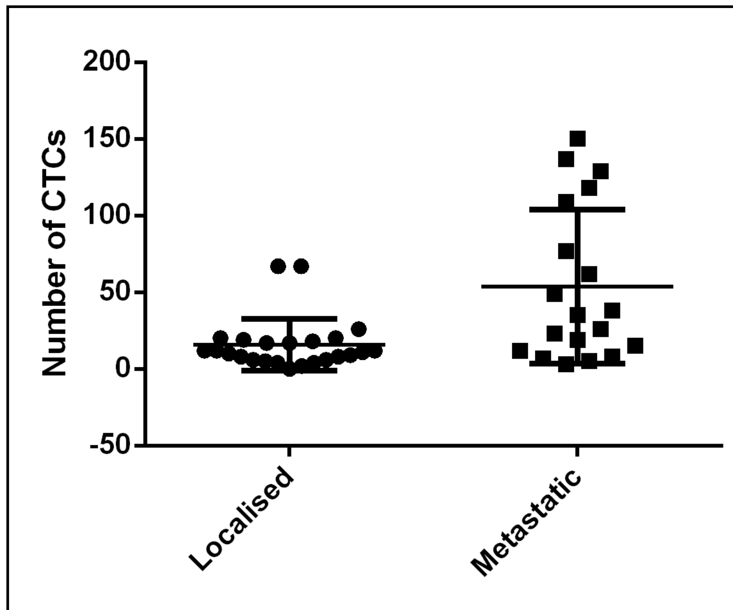


Figure 3. Circulating tumor cell count by stage. CTC processing post cryopreservation produced a higher mean CTC count in metastatic patients compared to the resectable patients (mean CTC in metastatic 53.8 vs resectable 15.8, p=0.0013).

Currently there are no established cut-offs for prognostic CTC numbers detected using the IsoFlux in gastroesophageal adenocarcinoma. Therefore we opted to divide our patients by their CTC counts, above versus equal or lower than the median CTC count, to test for any correlation with clinical outcomes. Patients with a high CTC count (>17) had a poorer overall survival (OS) than those with a lower CTC count (≤17) (Figure 4, median OS 2.8 vs 23.2 months, HR 4.4: 95%CI 1.7 – 11.7, p=0.0013).

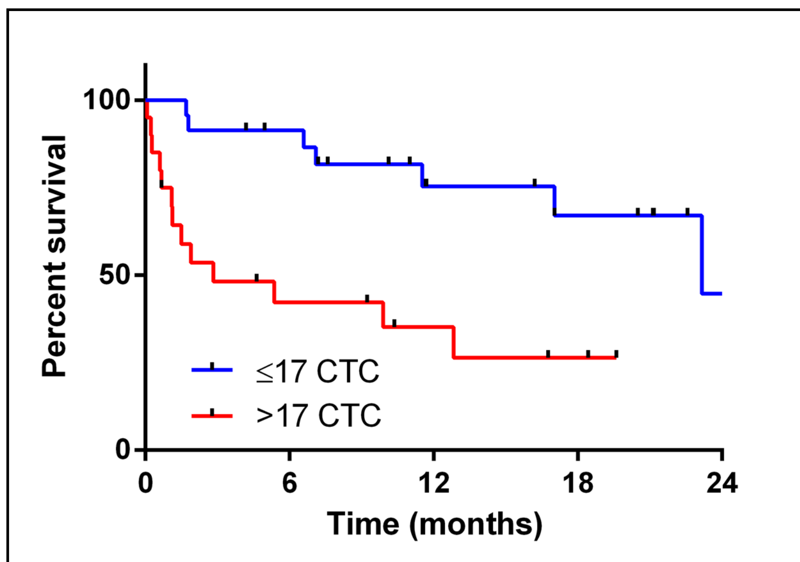


Figure 4: Overall Survival by CTC count. Patients with >17 CTCs isolated from cryopreserved specimens had a poorer overall survival compared to those with ≤17 CTCs (median OS 2.8 vs 23.2 months, HR 4.4: 95%CI 1.7 – 11.7, p=0.0013).

In multivariate analysis, after controlling for sex, age, stage, ECOG performance status, and primary tumor location, a high CTC count remained an independent prognostic factor associated with poor OS (Table 2, HR 3.7 95%CI 1.2 – 12.4, p=0.03). This association was stronger when the analysis was restricted to patients with metastatic disease (n=19, HR 5.5 95%CI 1.2 – 25.5, p=0.01), but not observed in patients with resectable disease (n=24, p=0.39), although a high CTC count (>17) was associated with a non-significant trend to shorter recurrence free survival in these patients (HR 3.1 95% CI 0.8 – 12.6, p = 0.09).

	Univariate		**Multivariate**	
Factor	**HR (95%CI)**	**P**	**HR (95%CI)**	**P**
CTC count (high vs low)	*4.4 (1.7 – 11.7)*	*0.001*	*3.7 (1.2 – 12.4)*	*0.03*
Age (≥65 vs <65 years old)	0.7 (0.3 – 1.8)	0.46	1.0 (0.9 – 1.1)	0.76
ECOG (2-4 vs 0-1)	*7.2 (2.2 – 23.7)*	*0.0002*	2.3 (0.5 – 10.1)	0.14
Sex (male vs female)	1.2 (0.4 – 3.8)	0.7	0.7 (0.2 – 2.1)	0.49
Stage (IV vs II-III)	*10.0 (3.3 – 30.8)*	*<0.0001*	*9.9 (2.9 – 33.8)*	*0.0003*
Primary tumor location (gastric vs oesophageal/GOJ)	0.3 (0.1 – 1.01)	0.05	0.4 (0.2 – 1.6)	0.22

Table 2: Univariate and Multivariate analysis for overall survival for Cohort 2 (n=43). Significant values are italicised. In both univariate and multivariate analysis, a high CTC count (>17) remained statistically significant as an independent factor associated with poorer overall survival. CTC – circulating tumor cell; ECOG – Eastern Cooperative Oncology Group performance status; GOJ – gastroesophageal junction

Most patients had some uPAR positive CTCs (40/43, 93.0%), however the proportion of uPAR positive CTCs was similar between patients with localised and metastatic disease (mean proportion uPAR positive CTCs 48.8% vs 47.7% respectively, p=0.89), and there was no association with survival outcomes (Supplementary Figure 1, median OS 17.0 vs 12.8 months, p=0.6).

4.4 Discussion

In this study we report the reliable isolation, immunocytochemical identification, and enumeration of gastroesophageal cancer CTCs from cryopreserved PBMCs using the IsoFlux platform. The included cohort is the largest reported study analysing cryopreservation of patient PBMCs for CTC detection. Our data confirms that CTCs isolated from cryopreserved samples remain an independent prognostic factor associated with overall survival.

The timely processing of patient samples for CTC isolation, usually is recommended within 24 h for most isolation methods[21], presenting significant logistical challenges for researchers and prohibits inclusion of patients from remote areas into clinical trials that would rely on CTCs as outcome measures. This is mainly because current methods of CTC analysis require significant expertise, instrumentation, time and laboratory resources, usually performed in specialised research centres. Protocols using isolation of CTCs from cryopreserved specimens, even though they require some basic processing and cryopreservation at the site of blood draw, offer many advantages, including the ability to biobank patient samples for prolonged periods of time before central processing. This would be a huge benefit for larger scale clinical trials as it would allow inclusion of geographically separated sites.

Previous work has shown that the immunochemical properties CK, EpCAM and CD45, central to the isolation and identification of CTCs, are not affected by cryopreservation and thawing[22,23]. In agreement, our results demonstrate a similar morphological and immunofluorescent profile between cryopreserved and fresh CTCs and leukocytes, suggesting current techniques are suitable for cryopreserved samples. This approach is further supported by other work showing close concordance in genetic alterations seen on paired fresh and frozen CTCs[23].

Given our previous findings that the uPA system is a clinically relevant biomarker in primary gastroesophageal cancer [18], we undertook and successfully probed for uPAR expression in CTCs derived from cryopreserved and fresh samples. We previously have shown that higher expression of uPA, uPAR and PAI-1 in the primary tumour is associated with higher risk disease and poorer prognosis however, in this study there was no correlation between CTC uPAR expression with disease parameters. This suggests that the selection of epithelial (EpCAM-positive) CTCs might have affected any correlation of uPAR with patient outcome, as CTCs that present mesenchymal phenotypes, such as uPAR expressing cells, can escape standard methods of isolation reliant on epithelial markers[24]. Indeed Vishnoi et al. has previously reported the isolation of subsets of EpCAM-negative, uPAR and integrin β1 positive breast cancer CTCs, which further supports the concept of CTC heterogeneity[25]. Ultimately, we have successfully stained for a novel biomarker, uPAR, which further supports our crypreservation method as a valid CTC isolation approach.

One important concern with cryopreservation is the potential for loss of CTCs due to cell loss during freezing, storage, or thawing. In a study by Nejlund et al, who cryopreserved buffy coats in dimethyl sulfoxide mixed with Roswell Park Memorial Institute 1640 medium, tumor cell recovery from cryopreserved spiked tumor cells in normal controls was variable, with up to a 40% tumor cell loss[22]. However in clinical samples using matched fresh and cryopreserved specimens from the same patient, there was no consistent loss of CTCs, with the variation in CTC enumeration similar to those seen in

paired fresh samples in other studies [2,22]. Friedlander et al found that cryopreservation of PBMCs had no significant effect on the cell recovery from patients with metastatic prostate cancer[23]. Similarly, Li et al found no significant loss of spiked tumour cells in cryopreserved PMBCs, but reported a longer elapsed time (greater then 2 hours) between blood draw and crypreservation reduced reproducibility of CTC measurement, and altered cell morphology[26]. We noted a small loss of CTCs associated with cryopreservation, however this was predominately in samples with large numbers of CTCs (>50), where loss of some CTCs is more acceptable than samples with low CTC counts. We noted samples with high numbers of CTCs were more prone to cell clumping despite benzonase. This is normally due to the release of viscous DNA from cell death on thawing, leading to aggregates which prevent accurate CTC counting. We speculate that the higher disease burden in these patients, coupled with a corresponding systemic inflammatory response, lead to poorer cell viability within the PBMCs of high CTC-count samples. Some loss of CTCs in these samples will have little impact for prognostic and down-stream biomarker analysis purposes. There was no significant loss of CTCs in samples where the total CTC count was ≤50 (p=0.06).

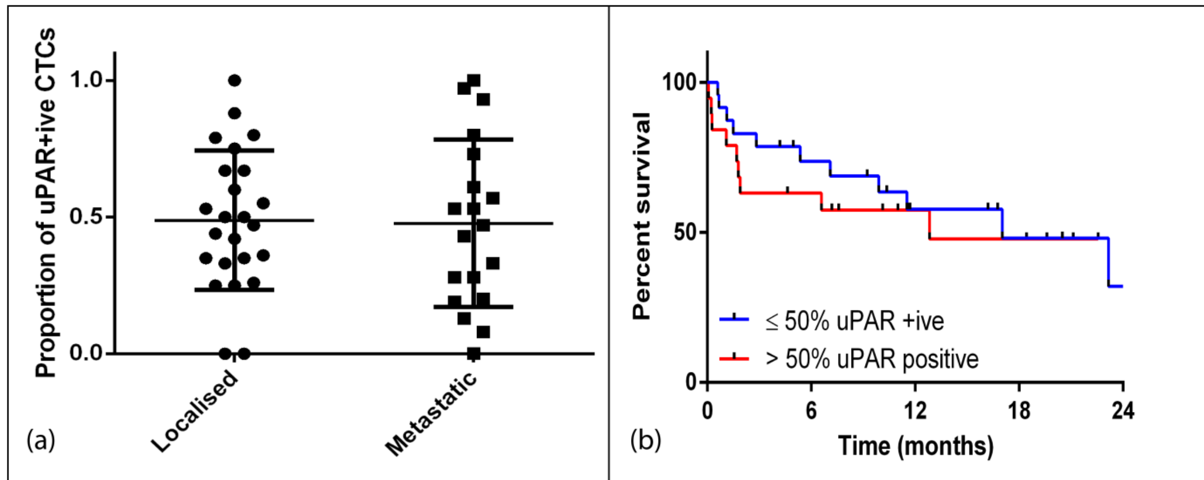
Similar to previously published work, we found that duration of cryopreservation was not correlated with number of isolated CTCs[22]. Moreover, we were able to isolate CTCs from specimens stored at -80C for over two years, suggesting cryopreservation is a suitable approach for long term projects that involve biobanking of patient samples.

Even using cryopreservation prior to CTC isolation, we found higher numbers of CTCs (median CTC count 17) and a higher number of patient samples with CTCs (98%) compared to other studies using EpCAM based CTC capture in gastroesophageal cancer[10-12,27]. The correlation of CTC numbers with disease progression implies that the CTCs we identified are indeed disease related. Increased CTC counts are consistent with the higher reported sensitivity of the IsoFlux system compared to other platforms, particularly in isolating CTCs with a lower expression of EpCAM[14-16]. Our results confirm, in the largest cohort of patients reported to date, that a high CTC count (>17) in cryopreserved specimen was an independent prognostic factor associated with poorer OS (HR 3.7). As expected from the minimal CTC loss during cryopreservation, these data indicate that indeed our method is suitable for delayed and centralised CTC analysis which could help recruiting patients for major clinical trials. In this setting it would be advantageous compared to fixation of blood which allows CTC processing delayed by only several days rather than long term biobanking. We are currently testing if cryopreservation is also able to overcome limitations associated with using fixative for molecular down-stream analysis of CTCs that involves nucleic acid extraction[4,5].

In conclusion, we have tested a robust PBMC cryopreservation protocol that allows successful CTC isolation even 2 years post freezing. Cryopreservation of CTCs is feasible, with a small loss of tumor

cells predominantly in samples with a high CTC load. Enumeration of CTCs from cryopreserved samples remained a clinically important prognostic biomarker. Cryopreservation may assist with the wider incorporation of CTC collection and analysis in biobanking, retrospective studies, and large international clinical trials, by facilitating specimen storage, bulk transporting, and batch processing. It may also help to develop diagnostic settings that can service even remote patients with diagnostic CTC data potentially relevant for their disease management.

Supplementary Figure 1: uPAR and CTCs (a) There was a similar proportion of uPAR positive CTCs in patients with localised and metastatic disease (mean proportion uPAR positive CTCs 48.8% vs 47.7% respectively, p=0.89) (b) Patients with >50% CTCs positive for uPAR had a similar overall survival compared to those with ≤ 50% uPAR positive CTCs (median OS 12.8 vs 17.0 months, p=0.60)



4.5 Appendix: Expression of uPAR on CTCs in gastroesophageal cancer

4.5.1 Background

A major challenge in CTC research is the development of an ideal marker, or combination of markers, to isolate and detect the rare CTCs within the large numbers of benign cells[1]. The CellSearch system - the only current FDA approved CTC system, uses positive immunomagnetic isolation of EpCAM (an epithelial cell marker) positive cells using anti human EpCAM antibody labelled magnetic beads. The cells are counterstained post enrichment with cytokeratins (a second epithelial marker to improve specificity), DAPI (nuclear stain), and CD45 (a leukocyte marker)[2]. The CTC is then identified with automated immunofluourescence microscopy, defined by an EpCAM/CK/DAPI positive and CD45 negative phenotype. Enumeration of CTCs using this approach have been shown to be clinically relevant prognostic biomarker in a range of cancers including breast[7], bowel[8], and prostate cancer[9], and is the most widely accepted definition of CTCs.

However there are increasing limitations recognised with this phenotype definition. CTCs undergoing epithelial-mesenchymal transition (EMT), an essential step in the passage of malignant cells into the blood steam for transit to distal metastatic sites, are known to downregulate EpCAM and CK which leads to a reduced sensitivity in detecting CTCs[28]. In addition, modelling studies suggest that 1 in 60 million CTCs using the standard phenotype form viable metastases[29], and there have been studies reporting long term (>22 years) persisting CTCs with no clinically visible disease[30]. Therefore there is a need to develop additional markers to improve sensitivity and specificity of CTC detection.

The uPA system is the key proteolytic pathway to facilitate invasion of cancer cells into stromal tissue (Section 1.2.3.1). uPAR has been identified as an important marker on CTCs in breast cancer, with uPAR+ CTCs enriched for stem cell pathways, as well as being able to adhere, proliferate and invade *in vitro* [25]. While there are no studies examining expression of the uPA system in gastroesophageal cancer CTCs, there are some compelling results supporting the importance of uPA system in disseminated tumour cells (DTC) in bone marrow. DTCs are thought to represent the fraction of CTCs capable of entering distant sites as the first step in establishing metastases [31]. Allgayer *et al* used CK18 to identify DTC in the bone marrow in 156 patients who had undergone a curative resection for localised gastric cancer. They found while overall CK18+ DTC was not associated with prognosis, the CK18+/uPAR+ subset was, suggesting uPAR identifies the critical subpopulation for establishment of metastases [32,33].

This study had two aims;

1. Correlate CTC uPAR expression with tumour tissue uPAR expression in patients with GOC and;
2. Determine the prognostic significance of CTC uPAR expression in GOC patients

4.5.2 Methods

CTC isolation and detection was performed as per Chapter 5 methods. It is important to note CTCs were isolated using the Isoflux system with EpCAM based CTC capture. CTC uPAR expression was characterised by proportion of CTCs (CK+/EpCAM+/DAPI+/CD45- cells) which stained positive for uPAR.

Eighteen patients from the CTC cohort (18/43, 42%) had FFPE tissue available for uPAR staining (see Chapter 4 for methods). uPAR expression in the tumour tissue was scored as followed: 0- No uPAR-positive cells; 1- Less than 1% uPAR-positive cells; 2- 1–5% uPAR-positive cells; 3- 5–10% uPAR-positive cells; 4- More than 10% uPAR-positive cells as previously reported for gastroesophageal cancer [34,35]. The tumour cell uPAR score at the invading edge was used for the tumour tissue uPAR score as data was limited for tumour core samples and is further justified by results from Chapter 3. Neutrophils were used as internal positive controls for uPAR staining on each slide.

4.5.3 Results

4.5.3.1 CTC and tissue uPAR expression

There was a trend to increased proportion of uPAR positive CTCs with increased tumour tissue uPAR score (Figure A1).

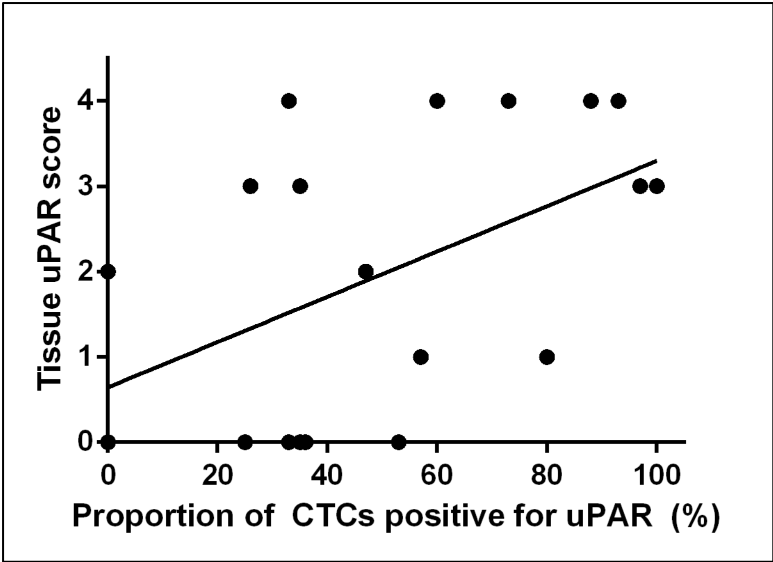


Figure A1: Correlation of tumour tissue uPAR score and proportion of CTCs positive for uPAR (n = 18; p=0.03).

Different cut-off levels for %CTCs positive for uPAR were analysed for significant association with tissue FFPE score (Figure A2). There was no significant association using a 50% cut-off (Mann-Whitney test, p=0.09). However, patients with ≥60% of CTCs positive for uPAR were more likely to have a higher tumour tissue uPAR score than patients with <60% CTCs positive for uPAR (mean tissue uPAR score 1.3 versus 3.3, p=0.0008).

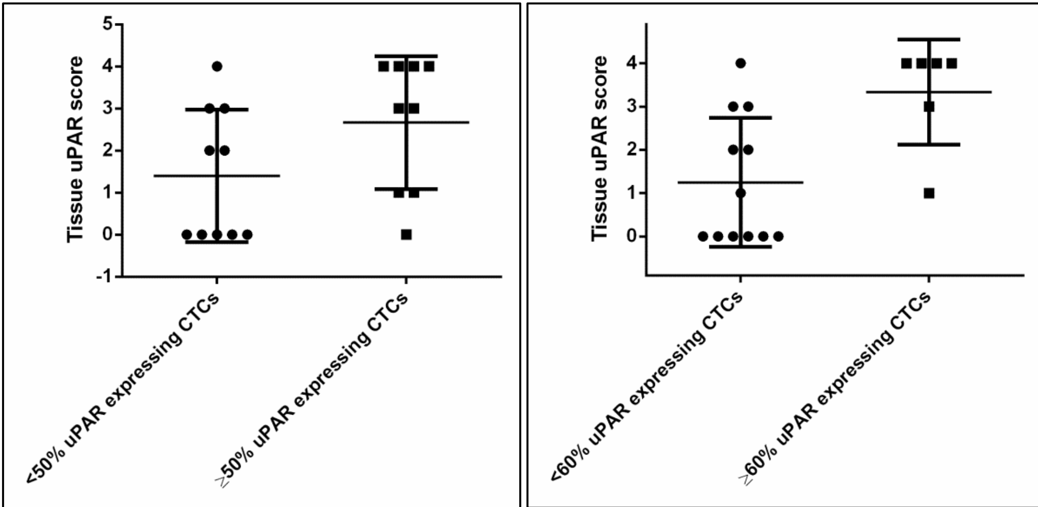


Figure A2: Association of proportion of CTCs positive for uPAR and tumour tissue uPAR score using a) 50% or b) 60% cut-off.

4.5.3.2 CTC uPAR expression as a prognostic biomarker

While CTC number was significantly associated with prognosis (Chapter 4), the proportion of CTCs positive for uPAR was not significantly associated with survival despite a variety of cut-offs tested. Absolute number of CTCs positive for uPAR (using 10 uPAR positive CTCs) had a non-significant trend for OS, but this appeared predominately driven by the total number of CTCs (Figure A3).

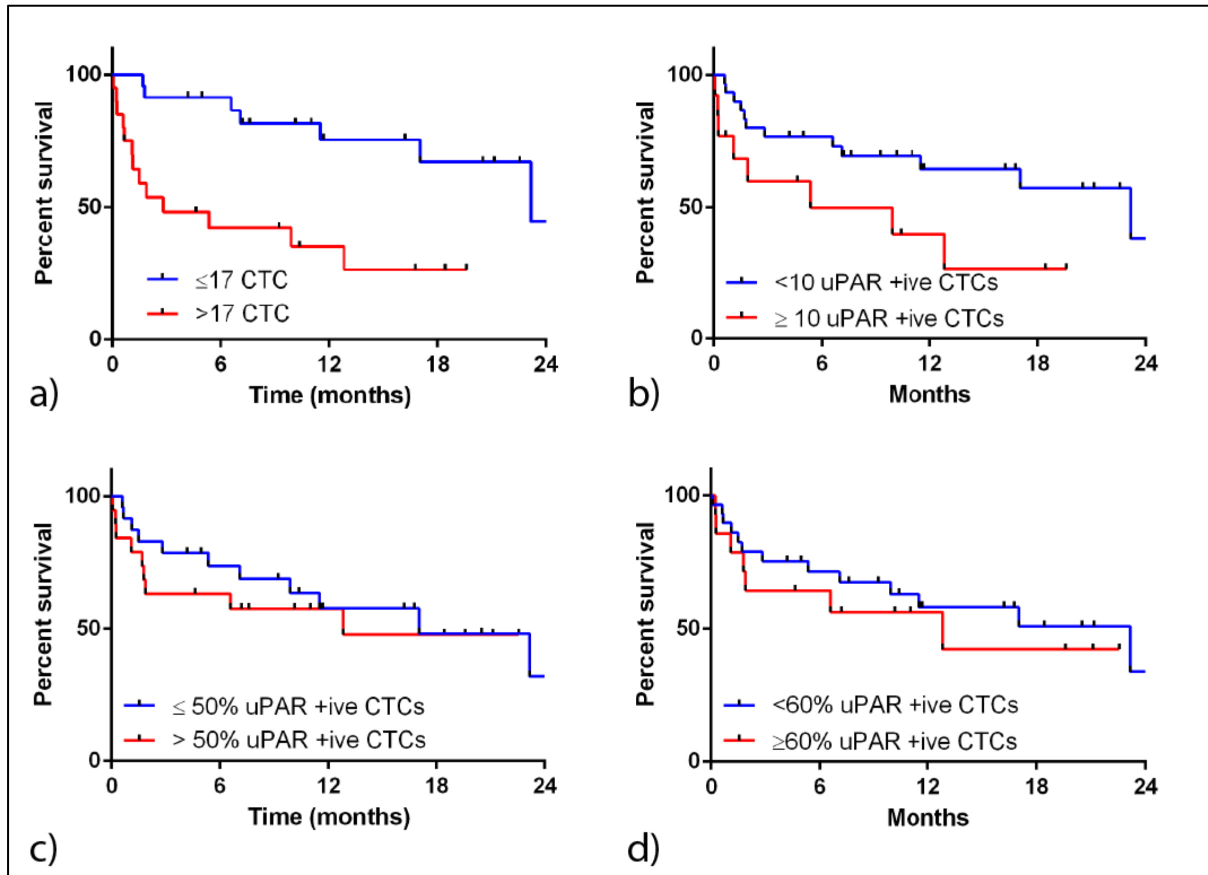


Figure A3: CTC counts and association with OS (n=43 for all analyses). a) Total CTC count is significantly associated with OS (see Chapter 5 for details) b) number of uPAR positive CTCs shows a non-significant trend with OS (p=0.06). There is no association with proportion of CTC uPAR positive with OS using any cut-off including c) 50% (p=0.6) or d) 60% (p=0.5).

4.5.4 Discussion

There are three principle findings from this study. Firstly, we found only a weak association between CTC uPAR expression and tumour tissue uPAR score, with patients ≥60% of CTCs positive for uPAR having a higher tissue uPAR score than those with <60% CTCs positive for uPAR. Secondly, while there were more CTC in metastatic disease than in localised disease, there was no significant difference in proportion of CTCs positive for uPAR. Thirdly, we did not find any association between CTC uPAR expression and OS. While there was a trend to poorer OS with < 10 uPAR+ CTCs compared to ≥10 uPAR+ CTC, this was driven predominately by the total CTC number.

The above conclusions are tempered by the selection bias of CTCs used in our study. We employed EpCAM immunomagnetic positive selection of CTC prior to staining and enumeration, leading to the omission of CTCs which have downregulated EpCAM as part of EMT. In the study by Allgayer *et al,* which demonstrated the strong association of uPAR expression on bone marrow DTC with risk of recurrent disease in resected gastroesophageal cancer [32], DTCs were defined solely by the CK+ phenotype. Although using EpCAM isolation improves the reliability and specificity of CTC isolation, it introduces the potential to miss important subsets of CTCs. Indeed, it has been postulated that the EpCAM- CTC population have the strongest potential to form distant metastases, and uPAR expression on these CTCs is a key determinate in breast cancer dormancy mechanisms [25]. We hypothesise that poor prognosis of tumours associated with high uPAR expression may be characterised by a higher proportion of EpCAM-/uPAR+ CTCs, which may be crucial for establishment of metastases. New strategies to account for these EMT-CTCs are emerging in recent years[36,37] and it would be interesting to use such CTC isolation methods to study uPAR expression on CTCs more comprehensively in gastric cancer.

4.5.5 Conclusion

There is no benefit to the addition of uPAR to the standard markers in EpCAM selected CTCs in GOC.

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Chapter 5

Circulating Tumour Cell Culture in gastroesophageal cancer

Circulating Tumour Cell Culture in gastroesophageal cancer

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Contribution of authors:

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MC, KV, MA – interpretation of results, manuscript revisions

SR, JW, AS – IHC

EM – RNA/DNA extraction, cytotoxic and radiotherapy assays

MT, SC – radiotherapy assays

TB, MR – concept and method development, interpretation of results, manuscript draft and revisions

Establishment and characterisation of two distinct and novel long term cell cultures derived from circulating tumour cells isolated from patients with metastatic gastroesophageal cancer.

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Abstract:
Most patients with advanced cancer have circulating tumour cells (CTCs) which can be detected in peripheral blood samples. CTC enumeration and profiling has been established as a valuable clinical tool in many solid malignancies including gastroesophageal cancer, particularly for prognostication and monitoring of treatment efficacy. A key challenge in CTC research is the very limited number of cells available for study. Long term CTC culture permits expansion of these rare cell populations for detailed characterisation, functional assays including drug sensitivity testing, and investigation of the pathobiology of metastases.

We report for the first time the establishment and characterisation of two long term CTC cultures from patients with gastroesophageal cancer. The two cells lines (designated JICTC and RFCTC) exhibit distinct genotypic and phenotypic profiles which are consistent with the tumours of origin. JICTC exhibits an EpCAM+, cytokeratin+, CD44+ phenotype, while RFCTC which was derived from a patient with metastatic neuroendocrine cancer, displays an EpCAM-, weak cytokeratin phenotype with strong expression of neuroendocrine markers. Both cell lines demonstrated rapid tumorigenic growth in immunodeficient mice.

Both cell lines have similar characteristics to their cancer of origin and show distinct differences to drug and radiation treatment. The establishment of these two cancer CTC lines will now enable a greater understanding of the biological processes driving gastroesophageal disease progression and act as a valuable tool to study drug responsiveness both *in vitro* and *in viv*o.

5.1 Introduction

Gastroesophageal cancers are among the most common and lethal cancers worldwide[1]. Most patients present with locally advanced or metastatic disease, or develop recurrent disease following curative surgery[2]. While many systemic treatment options are available, the prognosis of advanced gastroesophageal cancer remains poor, with median survival less than 1 year[3]. Greater than >90% of gastric and gastroesophageal junction cancers are adenocarcinomas, with gastrointestinal stromal tumours (GIST), lymphomas, and neuroendocrine tumours found in a small minority of cases[4].
Most patients with gastroesophageal cancer will require systemic treatment at some point in their disease management[3]. There is an increasing recognition of the limitations of using primary tumour features to guide systemic cancer treatment, due to tumour heterogeneity and the frequent disparity observed between primary and metastatic sites[5]. Metastatic biopsies are rarely undertaken however due to both inaccessibility of metastatic sites and procedure morbidity. Circulating tumour cells (CTCs) are the likely intermediates of metastasis dissemination of cancer, and as such, can be expected to include the subpopulations which are responsible for disease progression[6]. While CTC enumeration has an established prognostic role, the true promise of CTCs is to provide a ‘real time’ view of the cancer using only peripheral blood samples, avoiding the need of repeat invasive biopsies[7].
Moreover, while most cancer deaths are due to the haematological spread of metastases, research into the mechanism of metastasis initiation, formation, and propagation has been hampered by limited access to cancer cells within the various stages of the metastatic cascade. CTCs provide a unique window into the biology of cancer as it spreads through the blood stream. The rarity of CTCs compared to normal blood cells has provided significant technical challenges for sensitive but also specific isolation methods[8].

CTC culture provides an expanded cell population for expression analysis, functional assays, and drug sensitivity screening [9,10], and long term primary CTC cultures provide an ideal laboratory tool for the investigation of the biology of metastasis formation[11]. Establishment of long term primary CTC cell cultures has proved to be challenging. To date, despite intensive efforts, only several long term CTC cultures have been reported worldwide, including in colorectal[12], breast[13], and prostate[14] cancer, all established with modest success rates, with 1-16% of blood samples producing stable cultures. To improve culture rates, initial expansion of the CTC population using xenotransplation into immunodeficient mice prior to *in vitro* culture has been trialled [15,16].

In this current work we describe the establishment and characterisation of two novel CTC cell lines derived from patients with metastatic gastroesophageal cancer. To the best of our knowledge, this is the first report of long term CTC cultures established in gastroesophageal cancer.

5.2 Methods

5.2.1 Patient selection and blood collection

Peripheral blood samples were collected from patients with metastatic gastroesophageal cancer prior to treatment. Patients had histologically confirmed gastric or gastroesophageal cancer treated at the Illawarra Cancer Centre, Wollongong Hospital, NSW Australia. Informed consent was obtained from each patient prior to enrolment, and the study was approved by South Western Sydney Local Health District Human Research Ethics Committee (Project Number 15/072).

Initially 7.5 ml of blood was collected in EDTA tubes. This was increased to 15 ml after seventeen patients were enrolled for higher CTC capture to improve culture success rates. Blood samples were transported immediately at room temperature for CTC isolation. A second 7.5 ml EDTA blood sample was collected at the same blood draw from each patient for EpCAM based capture for CTC enumeration using the Isoflux System, and processed as per manufacturer instructions[17]. Enumerated CTCs were defined by the standard EpCAM/Cytokeratin/DAPI positive and CD45 negative phenotype[18].

5.2.2 CTC isolation and cell culture

Blood samples were incubated for 20 min with RosetteSep CTC Enrichment Cocktail with anti CD36 (Stemcell Technologies) prior to a density gradient separation with LeucoSep tubes (Stemcell Technologies) to isolate a peripheral blood mononuclear cell (PBMC) layer. We found excessive lymphocyte contamination prohibiting CTC growth with the RosetteSep Human CD45 Depletion Cocktail which was improved with the CTC Enrichment Cocktail. The PBMC layer was washed twice PBS, then immediately plated into 24 well ultra-low attachment plate (Corning) with serum free Advanced DMEM (ADMEM; Sigma Aldrich) supplemented with epidermal growth factor, fibroblast growth factor and N2 supplement, or ADMEM with 10% foetal calf serum (FCS) in hypoxic conditions. All media was supplemented with antibiotics (see Supplementary Table 1 for media formulations).

Patient 41 was noted to have marked peritoneal disease and gross ascites from gastric adenocarcinoma. At the same time as the blood draw for CTC isolation, 200 ml of ascitic fluid was collected from a peritoneal catheter. This sample was transferred immediately to the laboratory, washed twice in ADMEM with 1% antibiotic/antimycotic (Sigma Aldrich), and cultured as per conditions described above. Cultures derived from the CTC and ascites were maintained independently under identical conditions. For all subsequent cell culture and experimental assays, cells were maintained in ADMEM with 10% FCS and EGF.

5.2.3 Immunohistochemical analysis of patient samples, cell lines, and mouse xenografts

Expression of key proteins on the cell lines, mouse xenografts, and representative sections from the matching patient's tumour specimen were compared using immunohistochemistry (IHC). For cell lines, cells were collected, centrifuged with supernatant removed, then clotted with plasma and commercially prepared thrombin (Fibriprest Automate from Stago) to prepare a cell block. All samples were fixed in 10% formalin and then paraffin embedded, with 4 µm sections cut for staining. Antigen retrieval and development was performed on the fully automated Bond system according to manufactures instructions (See Supplementary Table 2 for antibody details), with positive controls included on each slide.

5.2.4 DNA and RNA extraction

Tumour and cell culture nucleic acids were extracted using AllPrep DNA/RNA FFPE Kit (80234, Qiagen) or AllPrep DNA/RNA/Protein Mini Kit (80004,Qiagen), respectively, according to the manufacturer's instructions. All samples were quantified using the NanoDrop (ND1000, Thermoscientific). RNA samples had A260/280 ratio between 1.7 and 2.3 (see supplementary Table 3).

5.2.5 Nanostring Analysis

Cell line RNA expression was explored and compared to the corresponding patient tumour and mouse xenograft using the Nanostring platform. 25 ng of RNA from fresh-frozen samples and 150 ng from FFPE samples were run on the NanoString nCounter Sprint system using the 770 gene PanCancer Pathways panel with additional cancer stem cell and proteolytic genes as per the manufacturer's instructions (NanoString Technologies). Results were analysed using the NanoString nSolver 3.0 and PanCancer Pathways Advanced Analysis Module, which normalizes gene expression to a set of positive and negative control genes built into the platform. Differential expression of genes from key pathways were compared between cell lines, with fold change and *P* values calculated using nCounter default settings. As recommended, genes whose expression levels were at or below the level of the negative controls were removed from analysis. With the remaining list of genes, a filter cutoff of $\geq \pm 2$ fold change and P value < 0.05 were used to identify the significant gene expression changes based on the nCounter analysis.

5.2.6 Short Tandem Repeat (STR) analysis

STR profiles for the cell lines and their matching patient FFPE tumour tissue were verified by the PowerPlexR 18D System, using the following 18 markers (seventeen STR loci and Amelogenin): D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, Amelogenin, vWA, D8S1179, TPOX, FGA, D19S433 and D2S1338. As per standard practice, cell

lines were considered to match if profiles are more than 80% identical to source patient sample.

5.2.7 Mouse xenograft tumourogenicity

For confirmation of tumourigenicity, 2x 10^6 cells from early passages of each cell line were injected subcutaneously into the flank of NOD *scid gamma* (NOD.Cg-Prkdc<scid>IL2rg<tm1Wjl>/ SzJAusb) mice. Mice were monitored for tumour growth and sacrificed when the tumour grew greater than 10 x10 mm, or the animal demonstrated signs of stress (such as >15% weight loss) or evidence of impedance of tumour on movement. Tumours were collected from the sacrificed mice for culture, RNA and DNA extraction, and histological analysis. For culture from xenografts, tumour tissues were cut into approximately 1 mm pieces and then incubated with Milteny tumour dissociation enzymes in ADMEM, after which the tumour homogenate was centrifuged, the pellet resuspended, and plated. Epithelial cultures derived from JICTC and JIASC xenografts grew rapidly as loosely attached cells and were easily separated from mouse stromal cells by mechanical pipetting at P0. These were designated as RFCTC-M and JICTC-M respectively, to denote the fact that they were passaged through mice. All procedures were carried out in accordance to the Australian Code for the Care and Use of Animal for Scientific Purposes 2013, and approved by the University of Wollongong's Animal Ethics Committee (study AE15/17)

5.2.8 Cytotoxic Assay

Approximately 10,000 cells were seeded per well in triplicate into a 96-well plate 48 - 72 h prior to drug treatment with carboplatin, etoposide, paclitaxel or oxaliplatin (obtained from excess patient stock from private hospital). Cells were incubated with serial dilutions of each drug for 72 h with drug vehicle (either water, 0.9% saline or DMSO, depending on drug solubility) dilution kept constant across all drug concentrations and controls (final concentration of 0.2%). The viability of cells were assayed using CellTitre 96® Aqueous One Solution Cell Proliferation Assay (Cat # G3581, Promega Corporation, Fitchburg, Wisconsin, USA) using a Spectromax 250 UV plate reader and Softmax Pro software (Molecular Devices, Sunnyvale, California, USA). Cell viability of treated cells were normalized against cells receiving vehicle controls. This data were analysed using a logarithmic sigmoidal dose–response curve using the variable slope parameter to determine IC_{50} (GraphPad Prism 6.0). (GraphPad Inc.). Data is presented as a mean ± standard deviation (SD) from ≥2 independent experiments.

5.2.9 Radiotherapy Assay

The sensitivity of the cells to radiation with drug pretreatment was also investigated using clonogenic survival as the radiobiological endpoint. Cells were first acclimated for at least a week in normoxic conditions, then plated into 12.5 cm^2 tissue culture flasks in regular cell culture media (as above) in

order to reach ~60% confluency 3 days later. Cells were then pre-sensitized with 0, 1, 2 or 5 µM Carboplatin for 48 h prior to exposure to 1 or 2 Gy X-Ray radiation. Cells were then passaged into triplicate tissue-culture petri dishes (100 mm x 20 mm Falcon BD; Pacific Laboratory Products) in 10 mL of ADMEM with 10% FCS, pencillin/streptomycin, EGF and l-glutamine, at different cell densities per dish (ranging from 1000 to 20000 cells per dish). After approximately 15 doubling times, petri dishes were washed with PBS and adherent cell colonies fixed and stained with a 1:3 crystal violet:ethanol solution. Colonies (≥ 50 cells/colony) were manually counted and presented as Mean Plating Efficiency (MPE; [number of colonies]/[number of cells plated]*100) and surviving fraction (SF; MPE of treatment group/MPE of control group) as previously described [19].

5.3 Results

5.3.1 Establishment of long term in vitro CTC cultures in patients with metastatic gastroesophageal cancer

A total of 41 blood samples were processed for CTC culture, with 23 samples processed using the optimised protocol (15 ml blood sample with negative selection using the CTC Enrichment Cocktail). CTC were detected in 38/40 samples (93%) by the Isoflux system (one sample unable to be processed for CTC enumeration due to clotted specimen), with ≥10 CTCs found in 22 (54%) of samples. Numbers of CTCs detected ranged from 0 – 150, with the mean number of CTCs 27.3 (summarised in Supplementary Table 4).

Long term CTC cultures were established from two patients by processing 15 ml blood samples using the optimised protocol (Table 1). The first was established from patient 20 (cell line RFCTC), who had a low CTC count of 3 by EpCAM based capture despite widespread nodal and bone metastases. Patient 20 had a distal oesophageal/gastroesophageal junction carcinoma diagnosed on endoscopy. The patient received concurrent chemoradiotherapy to the primary tumour and locoregional nodal disease as planned neoadjuvant treatment. Despite an excellent local response to chemoradiotherapy, the patient rapidly developed widespread metastatic disease including a dural metastasis causing spinal cord compression. At the time of CTC sampling the patient underwent resection of this metastasis, with histopathology demonstrating high grade neuroendocrine carcinoma, a rare and highly lethal subtype of cancer occurring in <1% of patients with gastrointestinal cancers [20] .
The second long term culture was established from patient 41 (cell line JICTC) who had a high EpCAM based CTC count (109). This patient presented with diffuse bone and peritoneal metastasis. Endoscopy demonstrated a large ulcerated gastric mass confirmed on biopsy to be a gastric adenocarcinoma. A matched culture was established simultaneously from the ascitic fluid sample from the same patient (JIASC). Unfortunately both patients progressed rapidly prior to receiving further treatment and passed away.

Table 1: Characteristics of the source patients of long term CTC cell lines.

Patient Number	Primary Tumour	Sites of metastatic disease at blood draw	Treatment prior to blood sampling	CTC count by Isoflux System (7.5ml sample)	Key protein expression of cell line
20	Distal oesophageal high grade neuroendocrine carcinoma	Bone Widespread nodal Hepatic	Chemoradiotherapy with carboplatin and paclitaxel to primary tumour and regional nodal disease	3	Synaptophysin + CGA+ CD56+ EpCAM - Cytokeratin weak/low
41	Gastric adenocarcinoma	Bone Peritoneal	Nil	109	EpCAM + Cytokeratin + CD44 +

In both CTC cultures, viable cell colonies were seen within 3 weeks. In the RFCTC culture a large number of residual CD45+ lymphocytes persisted for the initial 4 passages. Cell populations expanded rapidly, and have been maintained continuously for over 12 months to date. Once established, the cultures have been adapted to grow in a variety of conditions, including serum free media supplemented with various growth factors or with 10% fetal calf serum, hypoxic or normoxic atmosphere, or ultra low attachment (ULA) or standard culture vessels, and remain viable after freezing at various passages and thawing.

The cell lines display discrete *in vitro* growth characteristics. JICTC grows in long mucinous, loosely aggregated strands (Figure 1). These strands are weakly attached to the flask surface and require only gentle mechanical dissociation for passaging. Altering growth conditions (such as media) does not have any discernible effect on JICTC phenotype. In contrast, RFCTC grow as an adherent culture, requiring trypsinisation for passaging, although a loose adherent spheroid phenotype is inducible with a hypoxic environment and serum free media (Figure 2). Similar growth characteristics were seen in hypoxic and normoxic conditions once the cell line was established (data not shown).

5.3.2 CTC culture recapitulates the pathological characteristics of source patient

The two established CTC cell lines demonstrate markedly different phenotypes and protein and gene expression patterns which faithfully recapitulate patterns of the source tumour (Figures 1, 2 and 3). As discussed, cell line JICTC was established from a patient with metastatic gastric adenocarcinoma

with widespread liver and peritoneal metastases. Both the patient's primary tumour and JICTC demonstrated high grade appearances by hematoxylin and eosin (H+E) stain (Figure 1) with a high Ki67 (>80%). As expected in adenocarcinoma, both the tumour and cell line strongly expressed cytokeratins (CAM5.2; Figure 3), in particular CK-20 (Figure 1). The JIASC cell line expressed an almost identical phenotype to JICTC. Strikingly, the gastric cancer stem cell marker CD44, was strongly positive in JICTC, while JIASC were negative (Figure 1).

In contrast, RFCTC, established from a patient with high grade gastroesophageal neuroendocrine tumour, had only weak patchy cytokeratin staining, but as expected expressed high levels of the neuroendocrine marker synaptophysin, CD56, and Chromogranin A typical of this cancer (Figure 2). Protein expression was constant from cell line to mouse xenograft and subsequent cell culture created from the mouse xenograft (Figure 2). This cell line had an otherwise bland IHC profile, with no staining for epithelial or stem cell markers (Figure 2, 3). H+E staining showed a high grade poorly differentiated tumour with high Ki67 (80 – 100%) (Figure 2). Differing media (10%FCS or serum free media) did not change the phenotype detected by IHC (data not shown).

Neither cell line expressed mesenychmal markers (Vimentin or N-Cadherin) or urokinase plasminogen activator receptor (uPAR), a key receptor for the initiation of the proteolytic cascade (Figure 3). No cell line showed any CD45 expression at any stage confirming these cultures did not derive from lymphocytes (Figure 3).

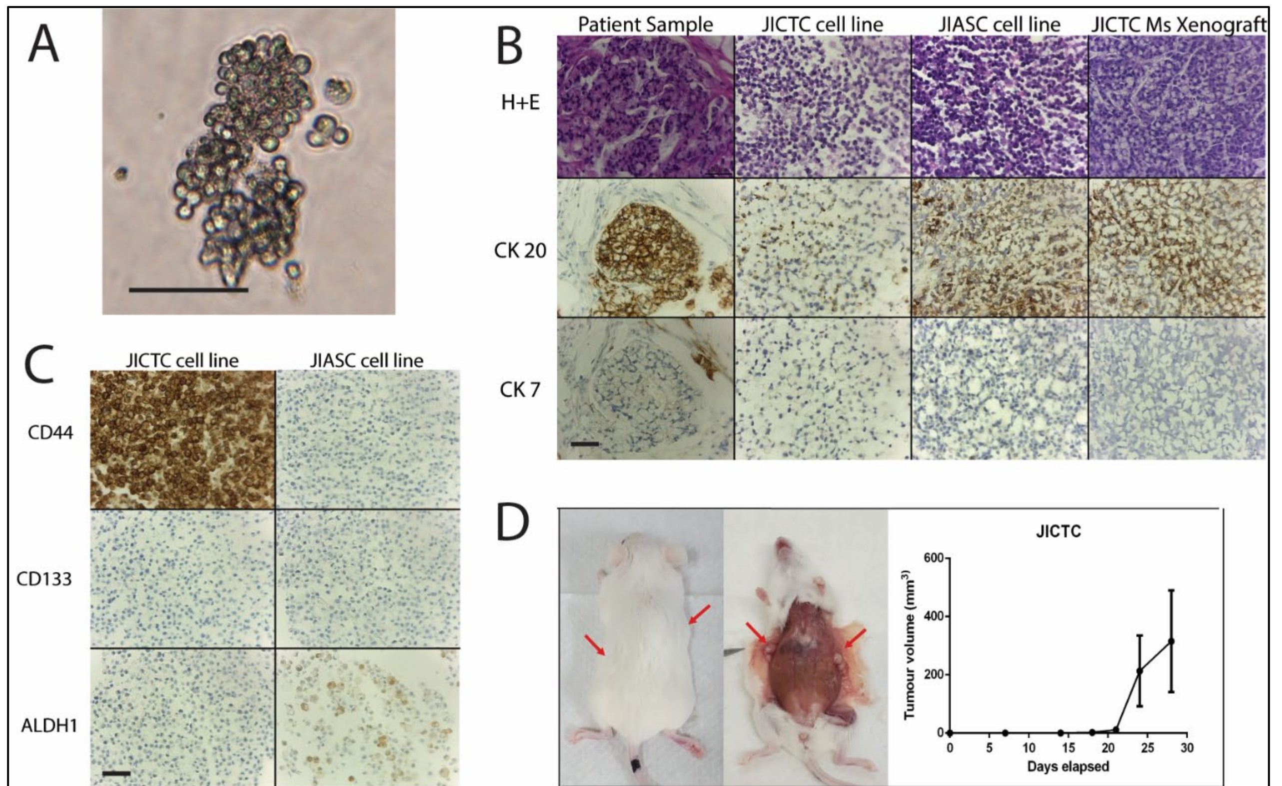

Figure 1: JICTC cell culture. (A) Representative images of the loose aggregates formed by JICTC. Scale bar 50 µm. (B) IHC analyses of primary tumour and cell line from patient 41 (cell line JICTC and JIASC). Both cell lines showed a very similar IHC profile, with strong pan-cytokeratin and CK-20 staining, and weak CK-7 staining, with an identical expression profile in tumours formed in the mouse xenograft. Scale bar 100 µm. (C) Expression of cancer stem cell markers in JICTC and JIASC. Scale bar 100 µm. (D) JICTC rapidly formed tumours in immunocompromised mice, with all mice reaching tumour endpoints within 4 weeks (mean ±SD of n= 4 mice).

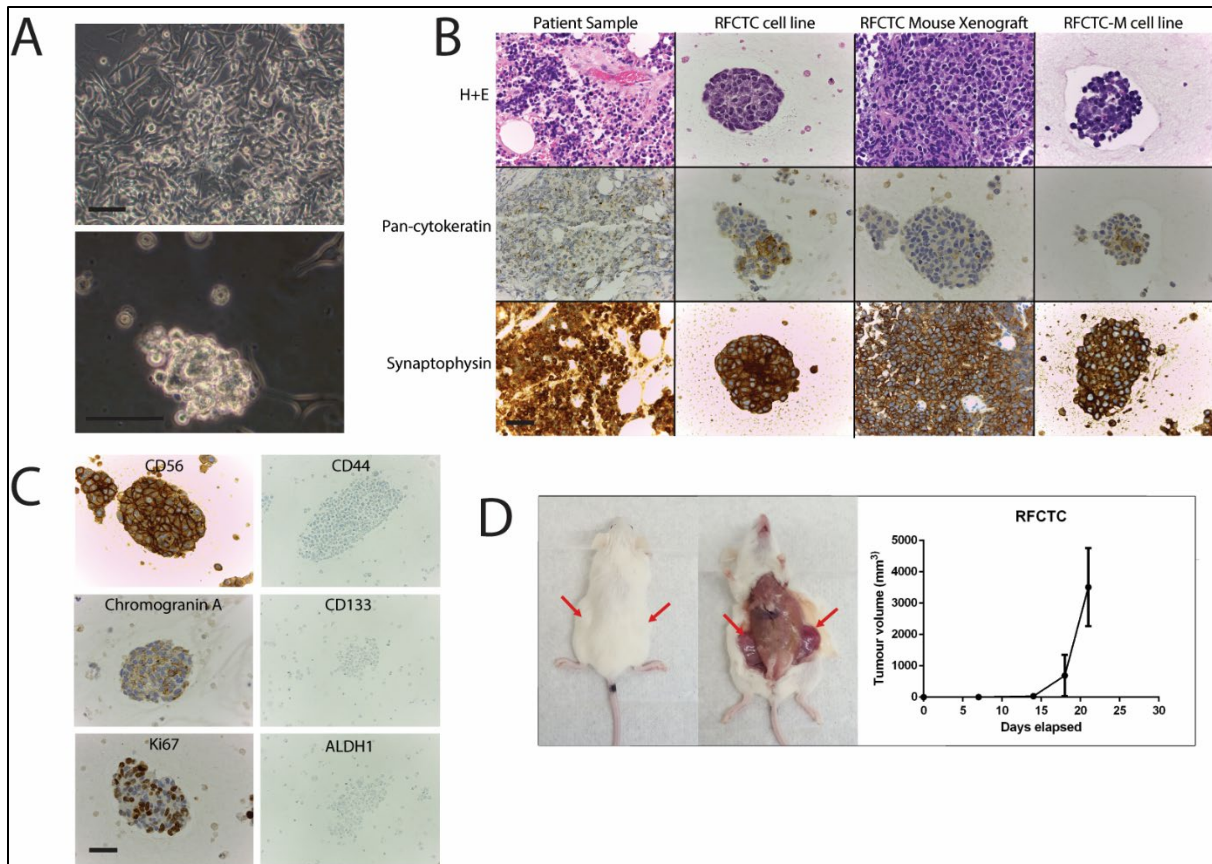

Figure 2: RFCTC cell culture. (A) Representative images of late passage (passage 40) RFCTC in varied culture conditions. While RFCTC grows attached to standard culture vessels in 10% FCS in normoxia and hypoxia (top image), a loosely adhered spheroid phenotype is inducible with serum free media and hypoxia (bottom image). Scale bar 50 µm. (B) IHC analyses of tumour, cell line, mouse xenograft, and cell line derived from mouse xenograft (RFCTC-M) from patient 20 showing stable and strong expression of the neuroendocrine marker synaptophysin, with consistent patchy cytokeratin positivity. Scale bar 100 µm. (C) IHC analysis of RFCTC showing strong expression of neuroendocrine markers (CD56 and CGA), a high Ki67, but no expression of CSC markers (CD44, CD133, ALDH1). (D) RFCTC rapidly formed tumours in immunocompromised mice, with all tumour endpoints reached within 3 weeks (data points are the mean ±SD of n= 4 mice).

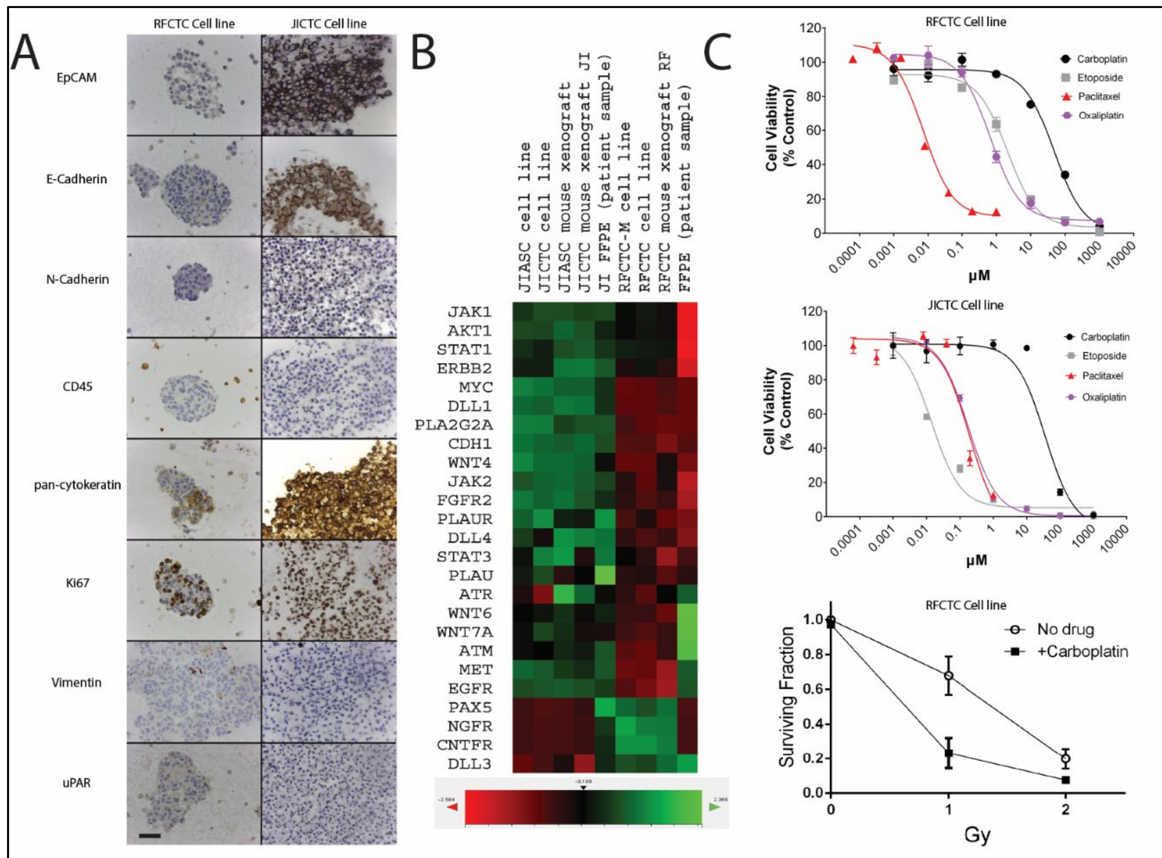

Figure 3: Comparison of JICTC and RFCTC (A) Immunohistochemical expression of key proteins. The distinct phenotypes of the two CTC cell lines are highlighted in this figure; JICTC expresses epithelial markers (EpCAM and E-Cadherin) as well as strong cytokeratin staining. In contrast RFCTC has no epithelial staining and weak/patchy cytokeratin staining. Both cell lines show a high Ki67 supporting a high proliferation rate. No CD45 staining was observed in any of the cell lines. Scale bar 100 µm. (B) Heatmap of RNA expression profiling of CTC cell lines (RFCTC, JICTC) with matching patient FFPE sample and mouse xenograft. Key highly differently expressed genes are displayed. JIASC cell line, established simultaneously from an ascites sample from the same patient is also reported (green = high expression, red = low expression) (C) Dose response curves for cytotoxic drugs on RFCTC (top) and (JICTC)**.** Cell culture for all analyses was performed under hypoxic conditions. Cell viability of treated cells were normalised against vehicle controls, and presented as mean ± standard deviation (SD) from ≥2 independent experiments. Cell survival of RFCTC determined by clonogenic assay (bottom). Cells were irradiated with or without carboplatin (1µM) pre-treatment. Surviving fractions of irradiated cells only (no drug) and drug only were normalized to non-irradiated non drug treated control. Each data point represents the means ±SD of at least two independent experiments performed in triplicate.

5.3.3 Mouse Xenograft tumourigenicity

Both cell lines rapidly formed tumours when injected subcutaneously into the flanks of immunodeficient mice. Tumour endpoints (>10 x10 mm) were reached within 3 weeks for JICTC and 4 weeks for RFCTC cell injection. IHC on excised tumours confirmed identical expression of human cytokeratins and cell surface protein markers to the original patient tumour and corresponding cell line (Figure 1 and 2). Cell cultures were established from the two CTC cell lines from the mouse xenograft (RFCTC-M and JICTC-M).

5.3.4 Tumour authentication

Detailed DNA analyses were limited by poor DNA quality from both source patient's FFPE samples. However, by STR analysis, 24/28 (85.8%) alleles of JIASC and JICTC were identical to the primary tumour from patient 41 confirming the source of the cell lines (see Supplementary Material). Despite multiple attempts, there was inadequate DNA extracted from FFPE samples of patient 20's tumour preventing analyses including STR. However we note the STR profile of RFCTC did not match any known cell lines in the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), or Garvan internal databases, demonstrating the that RFCTC is a novel high grade neuroendocrine cancer cell line.

5.3.5 CTC cell lines have distinct gene expression profiles

RNA expression analysis by Nanostring confirmed the IHC and STR findings showing two distinct CTC cell lines which reflect the source patient's tumour. While JICTC had a higher expression of CDH1 (encoding e-cadherin), RFCTC, as expected, had higher expression of genes for neuroendocrine markers including CNTFR, PAX-5 and NGFR[21-23].

Interestingly, differential expression analysis demonstrated JICTC, when compared to RFCTC, had a higher expression of genes known to be involved in *Helicobater Pylori* mediated carcinogenesis such as AKT[24], ETS2[25] and MYC[26], supporting endoscopic finding that patient 41's tumour was likely related to *H. Pylori* gastritis. There was also overexpression of genes encoding the gastric cancer stem cell markers CD44, ALDH1 and CD133, as well as key stem cell pathways such as NOTCH and WNT, including the notch delta-like ligands (DLL-1 and DLL-4) and PLA2GA, an important regular of metastases in gastric cancer and expressed with constitutively active Wnt [27-29]. JICTC also showed higher expression than RFCTC of targetable pathways including EGFR, FGFR2, HER-2, and MET, as well as key genes in the JAK/STAT pathway, genes which overexpression are frequently reported in gastric adenocarcinomas but not gastrointestinal neuroendocrine cancers[20,30,31].

RFCTC showed a high expression of DLL-3, which is known to be expressed on most high grade neuroendocrine cancers, particularly small cell lung cancer. Importantly it is targetable with the antibody-drug conjugate Rovalpituzumab tesirine[32,33]. RFCTC also showed a lower expression of key DNA repair kinases, including ATM and ATR. We did not observe other reported molecular features of high grade neuroendocrine cancers such as BCL-2 overexpression or Rb inactivation.

JICTC demonstrated a very similar RNA expression profile to JIASC. We did not find overexpression of CSC or epithelial to mesenchymal transition (EMT) genes in JICTC compared to JIASC.

Gene expression was preserved between cell lines and their corresponding mouse xenograft, with no significant difference in expression observed (Supplementary figure 1).

5.3.6 Cytotoxic and Radiosensitivity Assay

In vitro sensitivity of both CTC cell lines to the following cytotoxic drugs; carboplatin, etoposide, paclitaxel or oxaliplatin was evaluated using the MTS assay. JICTC and RFCTC demonstrated similar sensitivity to carboplatin and oxaliplatin, with both IC_{50} values (0.18 and 0.93 respectively) consistent with previously reported values for gastroesophageal cell lines (Table 2, Figure 3)[34]. Although patient 20 received chemotherapy with carboplatin and paclitaxel as a radiosensitiser prior to sampling for the CTC culture, RFCTC remained sensitive to both agents compared to the JICTC cell line.

Table 2: IC_{50} values for carboplatin, etoposide, paclitaxel and oxaliplatin on RFCTC and JICTC (all hypoxic conditions).

	IC_{50} (μM)	
Drugs	**RFCTC**	**JICTC**
Carboplatin	40.80 ± 2.94	33.40 ± 11.3
Etoposide	1.57 ± 0.40	0.04 ± 0.02
Paclitaxel	0.01 ± 0.03	0.55 ± 0.35
Oxaliplatin	0.93 ± 0.26	0.28 ± 0.13

We then investigated the sensitivity of each CTC cell line to radiotherapy with and without carboplatin sensitisation using the clonogenic assay. Unfortunately JICTC was found to be unsuitable for this assay due to the weak attachment to the culture plate. RFCTC displayed marked sensitivity to radiotherapy alone with a mean surviving fraction of 67% and 18% after 1Gy and 2Gy respectively. The prep-treatment of low dose carboplatin (1μM) significantly enhanced the effect of radiotherapy despite being much lower that then IC_{50} of carboplatin in RFCTC (40.8 μM) (Figure 3).

5.4 Discussion

We report the establishment and characteristation of two novel long term (>1 year, >40 passages) gastroesophageal cancer cell lines derived from CTCs. These two cell lines are genotypically and phenotypically distinct, reflective of differing tumour biology in the donor patients.

By IHC strong differences in the expression of key proteins were observed between the cell lines. Similar to other reported CTC lines, JICTC displayed an EpCAM+, cytokeratin (Cam5.2)+ , CD44+, phenotype. In contrast, RFCTC, isolated from a patient with neuroendocrine tumour, showed only patchy weak cytokeratin (Cam5.2) by immunostaining with strong expression of neuroendocrine markers (CD56+, synaptophysin+, chromogranin A+). RFCTC had no detectable EpCAM, mesenchymal (NCAD-, Vimentin-), or cancer stem cell markers (CD44-, CD133-, ALDH1-). This phenotype makes RFCTC a unique CTC cell line.

Overall both CTC derived cell lines as well as the ascites derived cell line recapitulated the phenotype of the source patient's tumour, highlighting that CTCs are a representative tumour source. It was interesting to detect differences in CD44, ALDH1 protein and STAT3 gene expression between the JICTC and JIASC, which probably reflects the different pathways of tumour cell dissemination. Of note, higher expression of cancer stem cell markers (CSC) and key stem pathways was also found on JICTC versus RFCTC, despite ALDH1, CD44, and CD133 reported as CSC markers in both adenocarcinomas and high grade neuroendocrine tumours[35,36]. Both demonstrated rapid *in vitro* and *in vivo* growth, had high grade histological appearance, and a high Ki67 (>80%), supportive of a stem cell phenotype. The RNA expression profiling confirmed these data, with each cell line's expression clustered with the source patient's tumour and the corresponding mouse xenograft.

STR analysis confirmed patient 41 as the source of JICTC. While we were unable to extract sufficient quality DNA from patient 20's FFPE tumour sample for any analysis, RFCTC's STR profile was unique.

Together with the confirmed tumourigenicity of the cell lines by the rapid development of tumours (within 4 weeks of inoculation) in all xenografted mice, these data confirm the establishment of two novel CTC derived immortalised cell lines. The tumours excised from the mice and the cell cultures (JICTC-M, RFCTC-M) derived from these tumours retained the overall RNA expression profile and IHC staining pattern seen in the corresponding cell line and source patient tumour. This highlights that these procedures, at least in short term cultures, do not grossly alter the original tumour characteristics, making CTC derived cell lines an excellent model to study tumour behaviour and response to treatment.

We also undertook limited cytotoxic and radiotherapy assays of the CTC cell lines based on standard cytotoxic drugs used for these cancers. We found drug sensitivities were generally similar to those previously reported for gastroesophageal cell lines[34]. We note the extreme sensitivity of RFCTC to radiotherapy, and the synergistic effect of carboplatin. This is consistent with the clinical experience of high grade neuroendocrine cancers, driven by low ATM and ATR expression, key DNA damage response enzymes and associated with increased response to radiotherapy[37-39]. Decreased ATM expression has been strongly associated with metastases in high grade neuroendocrine cancer from the gastrointestinal cancer[40]. RFCTC showing very low ATM and ATR gene expression together with the fact that the patient rapidly developed widespread metastatic disease indicates the capacity of CTCs for establishing metastases.

Multiple other potential therapeutic targets were identified by RNA expression profiling. JICTC had high expression of EGFR, FGFR2, ERBB2, and JAK/STAT pathway, suggesting potential sensitivity to treatments directed at these validated targets. Targeting ERBB2 has improved clinical outcomes in HER2 overexpressed gastric cancer, and ongoing clinical trials are exploring the use of FGFR2 and JAK inhibitors in these cancers[41-43]. Relative to JICTC,RFCTC had high expression of DLL3, known to be overexpressed in high grade neuroendocrine tumours, and targetable by Rovalpituzumab tesirine. These data show that CTC derived cell lines can be used to define personalised drug sensitivities for gastric cancer patients, an approach which has been shown to complement molecular profiling in personalising systemic treatments in cancer[44]More comprehensive drug sensitivity testing including drugs tailored against the suggested targets should be performed on these cell lines in the future.

Other studies have suggested a high CTC count (>300) is necessary for successful culture[12], however this is complicated with the inherent biases when selecting the isolation method and definition used for CTC enumeration. We employed the standard EpCAM based isolation and cytokeratin based CTC identification in the matched blood sample. Due to the biological differences discussed above, our successful cultures derived from samples with high (109) and low (3) CTC counts, subsequently developing EpCAM positive and negative cell lines, respectively. This finding highlights a key issue in the CTC field. While patient 20 has a low EpCAM+ CTC count, the successful establishment of a CTC cell line argues that this patient had a high number of EpCAM- CTCs with the ability to establish metastatic deposits. These biologically relevant cells are missed using standard CTC isolation techniques. Moreover, it is important to emphasise that these neuroendocrine CTCs would also not be well enumerated based on mesenchymal protein based CTC isolation, thus neuroendocrine CTCs likely still represent an understudied yet potentially highly aggressive population of CTCs. The negative selection used for CTC culture (CD45 depletion), rather than the positive selection used for CTC enumeration (EpCAM capture) was critical to detect these cells. The optimal isolation method of

CTCs continues to evolve in the face of these challenges and while more emphasis has been given to epithelial to mesenchymal transition (EMT) phenotype CTCs in recent years[45] the data presented here highlight that better detection of neuroendocrine CTCs need to be considered as well.

The matched culture derived from ascitic fluid (JIASC) simultaneously established from patient 41 as JICTC demonstrated an identical phenotype, with the exception of cancer stem cell expression. JICTC was strongly positive for CD44, a key CSC marker in gastric cancer[28], while JIASC was negative for CD44. This is consistent with other results showing that CTC cultures exhibit a stem cell phenotype. This further supports the notion that CTC cultures develop from the CTC population which are able to establish metastases[12,46 47].

In conclusion, we report the first two long term CTC cell lines developed from patients with metastatic gastroesophageal cancer. The two cell lines displayed distinct profiles which faithfully recapitulate the source patient’s tumour. Our results support the developing role of CTC culture as an essential laboratory resource for the understanding of the biology of metastases and importantly undertake personalised screening for therapeutic strategies.

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5.5 Supplementary Material

Supplementary Table 1: CTC culture media

	Supplier	Concentration
Serum Free Media		
Advanced DMEM/F12	Thermo Fisher Scientific	
N2 Supplement	Life Technologies Australia Pty Ltd	1x
Epidermal Growth Factor	Life Technologies Australia Pty Ltd	20ng/ml
Fibroblast Growth Factor	Life Technologies Australia Pty Ltd	10ng/ml
Penicillin Streptomycin Solution	Sigma-Aldrich	1X
L-gluatmine	Sigma-Aldrich	1x
10% Serum Media		
Advanced DMEM/F12	Thermo Fisher Scientific	
Foetal Bovine Serum	Bovogen	10%
Epidermal Growth Factor	Life Technologies Australia Pty Ltd	20ng/ml

Supplementary Table 2: Antibodies used in immunohistochemical analyses:

Antibody	**Supplier**	**Dilution**	**Retrieval Time (min)**
CAM5.2	Leica	1:100	20
CK-7	Leica	1:50	20
CK-20	Leica	1:100	30
E-Cadherin	Leica	1:25	20
HER-2	Leica	1:100	20
Ki67	Dako	1:40	20
Leukocyte Common Antibody (CD45)	Dako	1:500	20
VIM	Dako	1:500	20
CDX-2	Dako	1:50	30
CD56	Leica	1:1	20
CHROM	Leica	1:200	20
Synapatophysin	Leica	1:100	20
Urokinase Plasminogen Activator Receptor	Dako	1:100	60
N-Cadherin	Sigma-Aldrich	1:100	60

Supplementary Table 3: RNA quality indicators for nanostring analysis

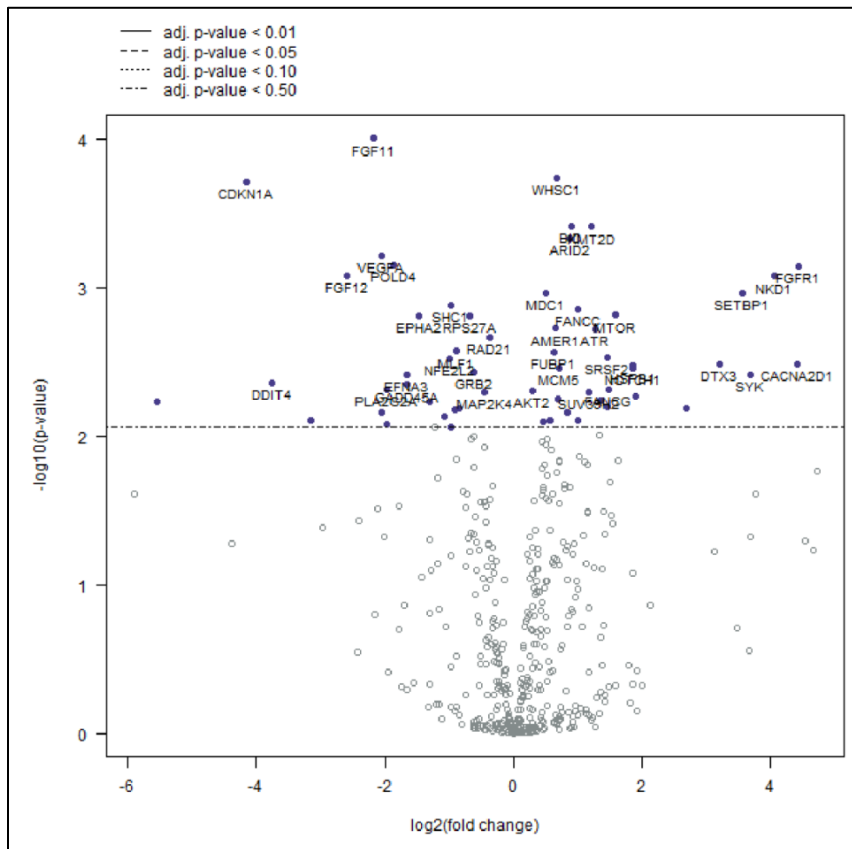
Sample #	Sample name	RNA concentration (ng/µl)	A260/280
1	Pt 41 FFPE (primary tumour, JI)	34.8	2.05
2	Pt 20 FFPE (metastatic deposit, RF)	128.1	1.66
3	JIASC (early passage)	431.8	2.07
4	JICTC (early passage)	365.1	2.06
5	RFCTC (early passage)	536.7	1.9
6	RFCTC hypoxic, late passage (p33)	1973	2.1
7	RFCTC normoxic, late passage (p33)	1348	2.09
8	RFCTC C spheroid (early floating?)	248	1.8
9	RFDH mouse xenograft(DHM1)	1384	2.12
10	RFCTCM (post mouse xenograft culture, early passage)	1517	2.09
11	JICTC mouse xenograft	1399	2.12
12	JIASC mouse xenograft	303	2.03

Supplementary Table 4: Patient samples used for CTC culture

Patient Number	CTC count by the Isoflux System in 7.5ml blood sample	Patient blood volume used for CTC culture (ml)	CTC enrichment cocktail	Long Term CTC culture established
1	150	7.5	CD45 only	
2	0	7.5	CD45 only	
3	38	7.5	CD45 only	
4	27	7.5	CD45 only	
5	12	7.5	CD45 only	
6	4	7.5	CD45 only	
7	2	7.5	CD45 only	
8	26	7.5	CD45 only	
9	49	7.5	CD45 only	
10	18	7.5	CD45 only	
11	18	7.5	CD45 only	
12	67	7.5	CD45 only	
13	62	7.5	CD45 only	
14	8	7.5	CD45 only	
15	1	7.5	CD45 only	
16	3	7.5	CD45 only	
17	4	15	CD45 only	
18	2	15	CD45 only	
19	13	15	CD45 and anti CD36	
20	3	15	CD45 and anti CD36	Yes (RFCTC)
21	15	15	CD45 and anti CD36	
22	NA*	15	CD45 and anti CD36	
23	0	15	CD45 and anti CD36	
24	118	7.5	CD45 and anti CD36	
25	26	15	CD45 and anti CD36	
26	5	7.5	CD45 and anti CD36	
27	19	15	CD45 and anti CD36	
28	7	15	CD45 and anti CD36	
29	1	15	CD45 and anti CD36	
30	1	7.5	CD45 and anti CD36	
31	6	15	CD45 and anti CD36	
32	5	15	CD45 and anti CD36	
33	16	15	CD45 and anti CD36	
34	15	15	CD45 and anti CD36	
35	14	15	CD45 and anti CD36	
36	1	15	CD45 and anti CD36	
37	2	15	CD45 and anti CD36	
38	137	15	CD45 and anti CD36	
39	77	15	CD45 and anti CD36	
40	12	15	CD45 and anti CD36	
41	109	15	CD45 and anti CD36	Yes (JICTC)

*CTC enumeration unable to be performed due to specimen clotting

Supplementary Figure 1: Differential RNA expression between cell line and corresponding mouse xenograft. We did not find a significant difference in expression for any genes (all results p<0.10).



Chapter 6

Clinical biomarkers in locoregional colon cancer

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DB – research proposal, concept development, database development and QC, data analysis, interpretation of results, manuscript draft and revisions

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Sidedness is prognostic in locoregional colon cancer: An analysis of 9509 Australian patients

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Abstract

Background/Aim: Right sided colon cancer (RsCC) is proposed to be a distinct disease entity to left sided colon cancer (LsCC). We seek to confirm primary tumour location as an independent prognostic factor in locoregional colorectal cancer.

Methods: All patients with stage I – III primary adenocarcinoma of colon were identified from the New South Wales (NSW) clinical cancer registry (2006 – 2013). Primary tumour location (RsCC vs LsCC) survival analyses were conducted using the Kaplan-Meier method, and adjusted hazard ratios for 5-year all-cause mortality (OS) and 5-year cancer specific mortality (CSS) were obtained using Cox proportional hazards regression.

Results: We identified 9509 patients including 5051 patients with RsCC and 4458 with LsCC. Patients with RsCC were more likely to be older, female, have a higher Charlson comorbidity index, and have worse tumour prognostic factors. In univariate analysis of all stages combined, those patients with RsCC had a worse overall survival (OS, HR 1.20 95%CI 1.11 – 1.29, p<0.0001), although this was not significant in the multivariate analysis (HR 0.96 95%CI 0.89 – 1.04, p=0.35). Stage I patients with RsCC had a trend to improved OS (multivariate HR 0.84 95%CI 0.69 – 1.01, p=0.07) and a significantly improved CSS (multivariate HR 0.51 95%CI 0.35 – 0.75, p=0.0006). In stage II patients with RsCC there was a significantly improved OS (multivariate HR 0.85 95%CI 0.75 – 0.98, p=0.02) and CSS (multivariate HR 0.59 95%CI 0.45 – 0.78, p=0.0002) compared to LsCC. In stage III patients, those with RsCC had a worse OS (multivariate HR 1.13 95%CI 1.01 – 1.26, p = 0.032) and a trend to worse CSS (multivariate HR 1.12 95%CI 0.94 – 1.33, p=0.22).
Conclusions: Primary tumour location is an important prognostic factor in locoregional colon cancer with an effect that varies by stage. RsCC is associated with lower all-cause mortality in stage II, and higher all-cause mortality in stage III.

6.1.1 Background

Colorectal (CRC) is a common and lethal malignancy, projected to account for 13% of all new cancer cases diagnosed in Australia in 2015, and 10% of Australian cancer deaths[1]. In recent years there has been increasing interest in identifying the differences between right sided and left sided colon cancer, and the potential for using this clinical marker as a surrogate marker of tumour biology, with the intent of improved personalisation of systemic treatments.

There is a growing body of evidence to suggest that right sided colon cancers (RsCC) follow a different disease process compared to left sided tumours (LsCC). The proximal and distal colons are physiologically separate, arising from distinct embryological origins, with differences in tumour genetics, histology, presentation, and clinical features[2-4]. Patients with RsCC are older, more likely to be female, have more comorbidities, with poorer tumour histopathological features[5-8].

Despite this, there is ongoing debate whether primary tumour location is an independent prognostic factor in colon cancer. Most, but not all studies have found poorer survival with RsCC[7-11]. A recent meta-analysis found a statistically significant worse overall survival in patients with RsCC, although there was significant heterogeneity seen due the spectrum of included study designs, disease stage, and limited information about treatment received by patients[12]. Tumour stage may play a role, with a large Surveillance, Epidemiology, and End Results (SEER) program study showing worse overall survival in Stage III RsCC patients, but not in Stage I or II [7], although these finding have been recently challenged by a propensity score matched analysis of the SEER database, which showed a better prognosis in RsCC patients[9].

This current study aims to use a prospectively collected database of Australian patients to determine whether primary tumour location is an independent prognostic factor in locoregional colon cancer, and compare our findings to the literature.

6.1.2 Methods

6.1.2.1 Patient Cohort

The New South Wales (NSW) clinical cancer registry contains demographic and clinical data for patients diagnosed or treated for cancer in NSW, covering approximately 30% of the Australian population. Data is collected from pathological laboratories, hospitals and oncology departments under mandatory notification of new cancer cases irrespective of treatment.

We identified all patients with Stage I, II or III colorectal cancer in NSW from Jan 2006 to 2013 (n=9509) as per third edition of the International Classification of Diseases for Oncology (ICD-O-3)[13].

The registry also contained adjuvant chemotherapy treatment details for a more limited group of patients with stage II and III disease (n=4102).

Mortality data, including cause of death, was obtained with linkage to the NSW registry of Births, Deaths and Marriages (BDM) by the Centre for Health Record Linkage (CHeReL)[14]. The censor data for survival data was 1st December 2014. Primary tumour location was defined right sided (caecum to transverse colon) or left sided (splenic flexure to rectosigmoid). Patients with rectal cancer were excluded from analysis due to the different treatment paradigm to colon cancer in locoregional disease. No data was available for cause of death in 935 patients (10.1%) which were therefore excluded from the cancer specific death analyses. Patients were deemed to have died as a result of colon cancer only if the underlying cause of death, rather than an associated cause of death, was coded as C18-20.

Comorbidity data was obtained by CHeReL linkage of the clinical cancer registry data to the Admitted Patient Data Collection (APDC). The APDC contains all admitted patient services provided by New South Wales Public Hospitals, Public Psychiatric Hospitals, Public Multi-Purpose Services, Private Hospitals, and Private Day Procedures Centres. Comorbidities of each patient were quantified using the Charlson comorbidity index which predicts mortality from a range of 22 comorbid conditions[16]. ICD-10 codes were extracted from admissions prior to diagnosis, then translated into a Charlson comorbidity index (modified for cancer) using methods previously described[15,16].

All data linkage was performed by the Centre for Health Record Linkage, with only de-identified information provided to the researchers. The data sources used for this study required ethical and data custodian approval to access, link (by an independent and approved authority) and release for research. Approval for this project was provided by the NSW Population & Health Services Research Ethics Committee (approval HREC/13/CIPHS/39).

6.1.2.2 Statistical analysis

Our primary outcome was all-cause 5-year overall survival (OS) stratified by stage, defined as death within 5 years of primary diagnosis of colon cancer on basis of dates recorded in the cancer registry and BDM databases. The secondary outcome was cancer specific 5 year survival (CSS) stratified by stage, as per cause of death encoded on BDM data. Median values for OS and CSS-OS and corresponding 95% CI were calculated using Kaplan-Meier methods. Unadjusted and multivariable Cox proportional hazards regression analyses were used to estimate the association between tumour location and survival and to calculate corresponding hazard ratios (HRs) and 95% confidence intervals (CIs). The following variables were included in the multivariate model: age, sex, Charlson

Comorbidity Index, TNM stage, year of diagnosis, grade, and adjuvant treatment (receipt and type of adjuvant treatment performed in subset of patients only). All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC).

6.1.3 Results

6.1.3.1 Patient Characteristics (*n=9509)*

The characteristics of the NSW cohort is summarised in Table 1. The mean follow up was 46 months (interquartile range 27 to 71months). At the end of 5 years of follow up, 2686 (28.2%) patients had died, with 913 reported deaths (34.0% of deaths) due to colon cancer. 22% of patients had stage I disease, 39% stage II, and 39% had Stage III. There were slightly more RsCC (53%) than LsCC (47%). Patients with RsCC were older (61% vs 47% older than 70 years) , more likely to be female (54% vs 42% female), had higher Charlson comorbidity indices (CCI, 40% vs 34% CCI $\geq$ 1), and had worse prognostic features including higher TNM stage (79% vs 76% stage II/III), and higher grade tumour (23% vs 11% poorly differentiated).

Table 1: Patient Characteristics (n=9509)

Characteristic		All Patients (%)	Right sided tumour (%)	Left sided tumour (%)	P value
TNM stage	I	2104 (22)	1055 (21)	1049 (24)	<0.0001
	II	3684 (39)	2059 (41)	1625 (36)	
	III	3721 (39)	1937 (38)	1784 (40)	
T stage	1	1526 (16)	715 (14)	811 (18)	<0.0001
	2	1030 (11)	558 (11)	472 (11)	
	3	5075 (53)	2741 (54)	2334 (52)	
	4	1868 (20)	1031 (20)	837 (19)	
N Stage	0	5788 (61)	3114 (62)	2674 (60)	0.06
	1	3065 (32)	1576 (31)	1489 (33)	
	2	656 (7)	361 (7)	295 (7)	
Grade	Well differentiated	1244 (13)	635 (13)	609 (14)	<0.0001
	Mod. differentiated	6648 (70)	3278 (65)	3370 (76)	
	Poorly Differentiated	1617 (17)	1138 (23)	479 (11)	
Age group	≤60	1925 (20)	798 (16)	1127 (25)	<0.0001
	61– 70	2423 (25)	1189 (24)	1234 (28)	
	71 – 80	2814 (30)	1600 (32)	1214 (27)	
	>80	2347 (25)	1464 (29)	883 (20)	
Sex	Male	4913 (52)	2317 (46)	2596 (58)	<0.0001
	Female	4596 (48)	2734 (54)	1862 (42)	
Charlson Comorbidity Index	0	5957 (63)	3027 (60)	2930 (66)	<0.0001
	1 – 2	5083 (22)	1172 (23)	911 (20)	
	3 – 4	1023 (11)	596 (12)	427 (10)	
	5	446 (5)	256 (5)	190 (4)	
Adjuvant Chemotherapy	None	1775 (19)	955 (46)	820 (40)	0.0002
	Fluorouracil based	1098 (12)	553 (27)	545 (27)	
	Oxaliplatin doublet	1233 (13)	568 (27)	665 (33)	
	Unknown*	5403	2975	2428	
Year Diagnosed	2006 – 2009	5018 (53)	2644 (52)	2374 (53)	0.38
	2010 – 2013	4491 (47)	2407 (48)	2084 (47)	
Totals		9509	5051 (53)	4458 (47)	

*not included in multivariate analysis in chemotherapy cohort.

6.1.3.2 5 year all-cause mortality by primary tumour location.

The observed 5 year OS for patients with RsCC was 66% (95% CI 65 - 67%) compared to 70% (95% CI 69 – 72%) for LsCC. Unadjusted survival analysis demonstrated a higher mortality with RsCC in all stages combined (Figure 1, univariate HR 1.20 95%CI 1.11 – 1.29, p<0.0001). When stratified by stage there was significant difference in OS seen only in stage III, with a higher mortality seen in RsCC (Figure 1, HR 1.46 95%CI 1.31 – 1.63, p<0.0001) (Figure 1).

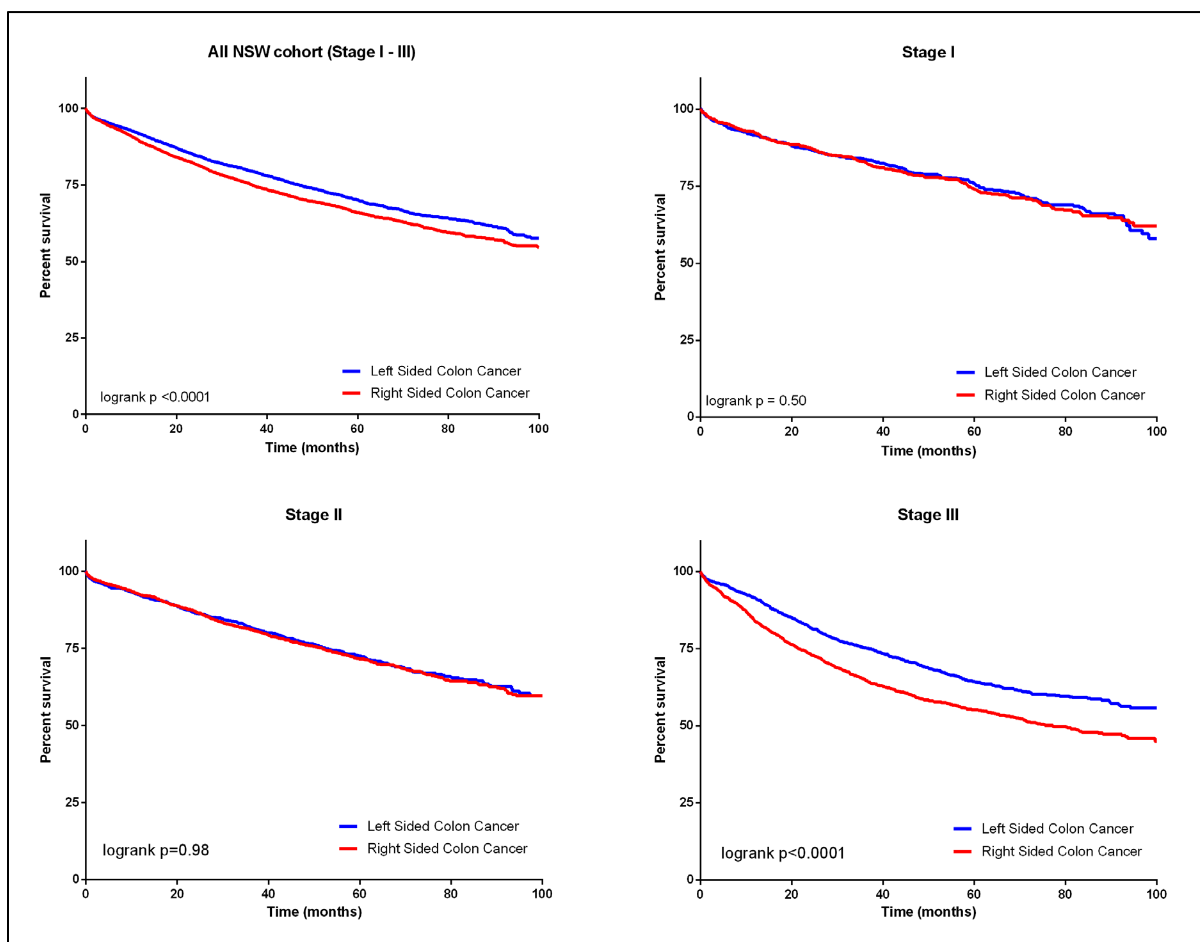


Figure 1: 5 year all-cause mortality by primary tumour location n= 9509 patients with 2686 deaths (Stage I = 2104 patients with 440 deaths, Stage II = 3684 patients with 883 deaths, Stage III = 3721 patients with 1363 deaths).

After adjusting for sex, age, comorbidities, stage, grade, and year of diagnosis there was no significant difference in OS between RsCC and LsCC in patients from all stages (multivariate HR 0.96 95%CI 0.89 – 1.04 p=0.35) (Table 2). When the multivariate analysis was stratified by stage, patients with RsCC had a trend to improved survival in stage I (HR 0.84 95% CI 0.69– 1.01, p=0.069), a statistically significant improved survival in stage II (HR 0.85 95%CI 0.75 – 0.98, p=0.02), but a shorter survival in stage III (HR 1.13 95%CI 1.01 – 1.26, p=0.03) (see Table 3.)

Table 2. Multivariate model for overall survival for NSW cohort (n=9509).

Characteristic		**Multivariate HR (95% CI)**
Sided	Left	1
	Right	0.96 (0.89 – 1.04)
Age	≤60	1
	61– 70	1.34 (1.15 – 1.56)
	71 – 80	2.23 (1.93 – 2.56)
	>80	3.97 (3.46 – 4.56)
Grade	Well differentiated	1
	Moderately differentiated	1.22 (1.06 – 1.39)
	Poorly Differentiated	1.87 (1.60 – 2.17)
TNM stage	I	1
	II	1.05 (0.96 – 1.21)
	III	2.00 (1.80 – 2.24)
Sex	Male	1
	Female	0.90 (0.83 – 0.97)
Charlson Comorbidity Index	0	1
	1 – 2	1.64 (1.49 – 1.79)
	3 – 4	1.81 (1.62 – 2.03)
	5	3.02 (2.63 – 3.46)
Year Diagnosed	2006 – 2009	1
	2010 - 2013	0.98 (0.90 – 1.06)

HR – Hazard Ratio; CI – confidence interval.

Table 3. Univariate and multivariate Hazard Ratios for NSW cohort (n=9509) stratified by stage. Statistically significant values in bold.

		Overall Survival HR (95% CI)		Cancer Specific Survival HR (95% CI)	
		Univariate	Multivariate*	Univariate	Multivariate*
All Patients	Left Sided	**1**	1	1	**1**
	Right Sided	**1.20 (1.11 – 1.29)**	0.96 (0.89 – 1.04)	1.03 (0.91 – 1.18)	**0.84 (0.73 – 0.96)**
Stage I	Left Sided	1	1	**1**	**1**
(n=2104)	Right Sided	1.03 (0.91 – 1.18)	0.84 (0.69 – 1.01)	**0.66 (0.45 – 0.95)**	**0.51 (0.35 – 0.75)**
Stage II	Left Sided	1	**1**	**1**	**1**
(n=3684)	Right Sided	1.002 (0.88 – 1.14)	**0.85 (0.75 – 0.98)**	**0.68 (0.52 – 0.88)**	**0.59 (0.45 – 0.78)**
Stage III	Left Sided	**1**	**1**	**1**	1
(n=3721)	Right Sided	**1.46 (1.31 – 1.63)**	**1.13 (1.01 – 1.26)**	**1.43 (1.21 – 1.69)**	1.12 (0.94 – 1.33)

*following variables were used in the multivariate analysis: age, sex, year diagnosed, Charlson Comorbidity Index, TNM stage, grade.

6.1.3.3 Cancer specific survival (CSS) primary tumour location

The 5 year cancer specific survival (CSS) was similar for RsCC (89%; 95%CI 88 – 90%) and LsCC (89%; 95%CI 87-90%). Unadjusted CSS analysis did not show a significant difference between RsCC and LsCC in all stages combined (Figure 2, univariate HR 1.03 95%CI 0.91 – 1.18, p=0.64). When stratified by stage, there was a significantly improved CSS seen with RsCC in stage I (HR 0.66 95%CI 0.45 – 0.95, p=0.024) and stage II (HR 0.68 95%CI 0.52 – 0.88 p=0.0032), but a significantly poorer survival for stage III patients (HR 1.43 95%CI 1.21 – 1.66, p<0.0001) (Figure 2, Table 3).

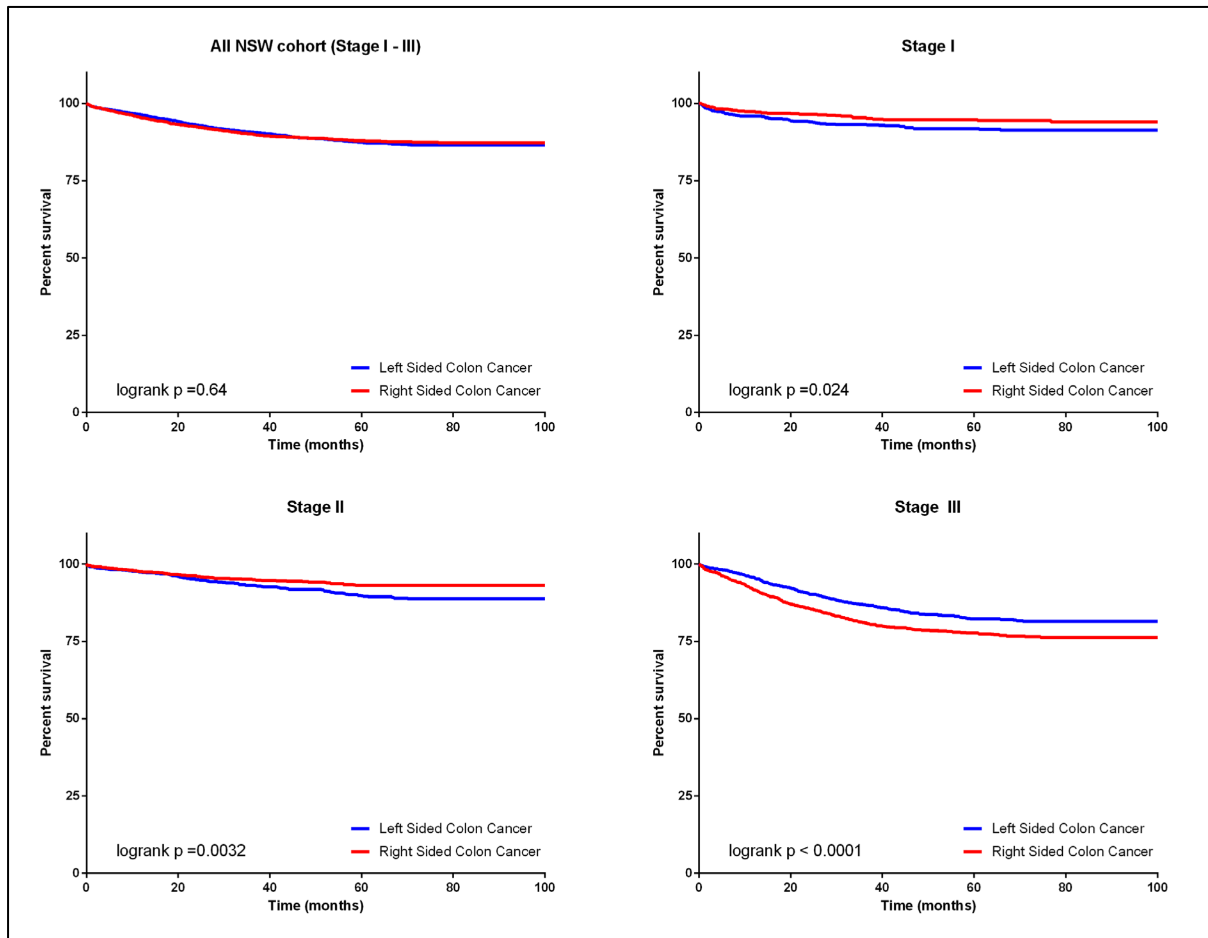


Figure 2: 5 year cancer specific mortality by primary tumour location n= 9509 patients with 2686 deaths (Stage I = 2104 patients with 116 deaths, Stage II = 3684 patients with 224 deaths, Stage III = 3721 patients with 573 deaths).

In the multivariate analysis, after adjusting for sex, age, comorbidities, stage, grade, and year of diagnosis, patients with RsCC had a statistically significant improved CSS in all stages combined (HR 0.84, 95%CI 0.73 – 0.96, p=0.011), and for stage I (HR 0.51 95%CI 0.35 – 0.75, p=0.0006) and stage II (HR 0.59 95% CI 0.45 -0.78, p=0.0002) patients, but a trend to worse survival in stage III (HR 1.12 95%CI 0.94 – 1.33, p=0.22) (Table 3).

6.1.3.4 Effect of adjuvant chemotherapy

Adjuvant treatment details were available for 1631 (44%) of patients with stage II and 2441 (66%) of patients with stage III disease (4102 patients total). Most patients in stage II disease did not receive adjuvant chemotherapy (72%), with only a minority receiving fluorouracil monotherapy (24%) or an oxaliplatin doublet combination (usually FOLFOX, 5%). In contrast, the majority of patients with stage III disease received adjuvant chemotherapy (75%), with 28% treated with fluorouracil monotherapy, and 47% with an oxaliplatin/ fluorouracil doublet. Higher TNM-substage was associated with treatment with oxaliplatin doublet within both stage II ($p<0.0001$) and III ($p=0.0001$). Consistent with current practice no patients received adjuvant treatment with monoclonal antibodies. Patients with RsCC were less likely to receive adjuvant chemotherapy ($p=0.0002$, Table 1) despite higher risk tumour features. Adjuvant chemotherapy improved survival in both RsCC (univariate OS HR 0.68; 95%CI 0.58 – 0.80) and LsCC (univariate OS HR 0.48; 95%CI 0.40 – 0.58, supplementary figures 1 and 2).

Inclusion of the adjuvant chemotherapy regimen into the multivariate model did not alter the effect of primary tumour location, although the results for RsCC in stage II disease became non-significant (multivariate OS HR 0.86 95%CI 0.69 – 1.09 $p=0.19$; multivariate CSS HR 0.67 95%CI 0.43 – 1.04, $p=0.07$, table 4). Patients with RsCC in stage III colon cancer continued to have a significantly inferior OS compared to LsCC even after adjustment for all above factors including receipt and type of adjuvant chemotherapy (multivariate OS HR 1.29 95%CI 1.11 – 1.50 $p=0.0012$; multivariate CSS HR 1.16 95%CI 0.92 – 1.47, $p=0.22$, table 4). When analyses were restricted to only those stage III patients who received adjuvant oxaliplatin doublet chemotherapy (n=1233), RsCC remained associated with a poorer OS (univariate OS HR 1.8 95%CI 1.4 – 2.4, $p<0.0001$).

Table 4. Multivariate model for overall survival for chemotherapy cohort (n=4102).

Characteristic		Stage II (n=1631) Multivariate HR (95% CI)	Stage III (n=2441) Multivariate HR (95% CI)
Sided	Left	1	1
	Right	0.86 (0.68 – 1.09)	1.29 (1.11 – 1.50)
Age	≤60	1	1
	61– 70	1.90 (1.20 – 2.99)	1.21 (0.94 – 1.54)
	71 – 80	2.97 (1.92 – 4.58)	1.81 (1.43 – 2.30)
	>80	5.92 (3.82 – 9.19)	2.00 (1.54 – 2.60)
Grade	Well/mod differentiated	1	1
	Poorly Differentiated	1.43 (1.08 – 1.90)	1.49 (1.26 – 1.75)
TNM stage	IIIa	1	1
	IIIb	2.20 (1.71 – 2.82)*	1.79 (1.33 – 2.43)
	IIIc	-	3.86 (2.84 – 5.24)
Sex	Male	1	1
	Female	0.85 (0.68 – 1.07)	0.94 (0.82 – 1.10)
CCI	0	1	1
	1 – 2	1.42 (1.09 – 1.52)	1.15 (0.96 – 1.38)
	3 – 4	1.60 (1.12 – 2.28)	1.20 (0.94 – 1.53)
	5	2.31 (1.45 – 3.69)	1.83 (1.36 – 2.46)
Year Diagnosed	2006 – 2009	1	1
	2010 - 2013	0.99 (0.79 – 1.26)	1.00 (0.86 – 1.17)
Adjuvant	Nil	1	1
Chemotherapy	Fluorouracil monotherapy	0.79 (0.51 – 1.10)**	0.48 (0.40 – 0.57)
	Oxaliplatin doublet	-	0.38 (0.27 – 0.42)

* IIa vs IIb/IIc, ** chemotherapy vs no chemotherapy

HR – Hazard Ratio; CI – confidence interval; CCI – Charlson Comorbity index.

6.1.4 Discussion:

There are well established differences in patient demographics, tumour factors and clinical presentation between RsCC and LsCC[7,9,10,17,18]. However it remains uncertain whether primary tumour location is an independent prognostic factor in locoregional colon cancer.

The strongest evidence comes from a recent meta-analysis of 66 studies including 1,437,846 patients which showed LsCC is associated with a significantly reduced risk of death compared to RsCC (HR 0.82; 95%CI 0.79 – 0.84, P<0.01)[12]. This study included all stages of colon cancer and found that, based on meta-regression, the effect of primary tumour location was independent of stage, race, year of study, and quality of study.

It is important to consider the limitations of the above meta-analysis. Firstly, there was significant heterogeneity seen in the results (I^2=93%), which is likely due to the variety of included study

designs, differing multivariate covariates from source studies, and patient populations, with the estimate derived from overall populations with no stratification by stage.

Secondly, while most of the included studies controlled for tumour factors (such as stage and grade), and patient demographic factors (eg., age, sex), only three studies included a comorbidity index in the multivariate model[7,17,19], and only 21% (14 of 66 studies) included performance status. RsCC is more likely to occur in older patients who have more associated comorbidities[17], and the substantial imbalances in the baseline characteristics between LsCC and RsCC patients in these trials may be an unmeasured confounder which explains the improved survival with LsCC. This issue has been directly addressed by Warschkow *et al* who, in order to minimise confounding, used propensity score matching to analyse survival in RsCC versus LsCC in 91,416 patients with stage I-III colon cancer from the SEER database. These authors showed that RsCC had a better OS (HR 0.89, p<0.001) and CSS (HR 0.71, p<0.001) in stage I and II, but a similar prognosis in stage III (OS HR 0.99, p = 0.49; CSS HR 1.04, p=0.129).

Our current study, using a large series of Australian patients from a prospectively collected database, and controlling for patient factors (including comorbidities), tumour factors, and adjuvant chemotherapy, confirmed previous studies showing that RsCCs are more likely to have a more advanced stage (p<0.0001) and grade (p<0.0001), and occur in older patients (p<0.0001) with more comorbidities (p<0.0001). Despite higher risk tumour features, patients with RsCC are less likely to receive adjuvant chemotherapy (p<0.0001) or oxaliplatin doublet chemotherapy (p=0.0002).
In the survival analysis, patients with RsCC have a lower all-cause mortality in stage II (HR 0.85, p=0.02), but a higher mortality in stage III (HR 1.13, p=0.032). Moreover, patients with RsCC had an improved 5-year CSS in Stage I (HR 0.51,p=0.0006) and Stage II (HR 0.59, p=0.0002), and a trend to inferior CSS in Stage III.

As adjuvant chemotherapy has been shown to have a larger benefit in RsCC than LsCC[20], we subsequently undertook further multivariate analysis in a subset of patients with known adjuvant chemotherapy protocols to validate our findings. Adjuvant chemotherapy improved survival in both RsCC and LsCC. We found incorporation of adjuvant chemotherapy into the multivariate model did not alter the effect of primary tumour location. Although definitive conclusions were limited in stage II as chemotherapy regimens where only available in 44% of patients, there were similar hazard ratios showing improved OS and CSS with RsCC (multivariate HR 0.86 and 0.67 respectively), although statistically non-significant in the chemotherapy cohort. In stage III, where chemotherapy data was available for the majority of patients (66%), the results of multivariate analysis was very similar to overall cohort, with a significantly higher all-cause mortality with RsCC (HR 1.29, p = 0.0012) and trend to higher cancer specific mortality (HR 1.16, p=0.21).

Our findings are consistent with the results of Wiess *et al* [7], a large multivariate retrospective analysis of 53,801 patients from the SEER database linked to Medicare data, and controlled for comorbidities using Hierarchical Condition Categories risk score. Similar to our findings, in multivariate analysis, patients with RsCC had a non-significant trend to lower mortality in stage I (HR 0.95, p=0.21), a lower mortality in stage II (HR 0.92, p<0.0001), but a higher mortality in stage III (HR 1.12, p<0.001), and a non-significant difference in mortality overall (HR 1.01, p=0.60). This stage dependant effect, with an improved survival in RsCC in stage II, but higher mortality in stage III, has been reported by multiple other series[8-10,18,21].

The cause of the demonstrated inconsistent effect of primary tumour location by stage is unclear. Our study, and the quoted literature, are retrospective analyses of large population databases, and are susceptible to the inherent bias of confounding associated with this study design. However an alternative explanation to consider is the increasingly described differences in tumour biology between RsCC and LsCC. RsCCs are more likely to have adverse histological features (such as advanced T stage, higher grade, or lymophvascular invasion) and mucinous histology[2,22-24]. Perhaps more importantly, there are also marked differences in the molecular profile between these tumours[25]. RsCC has a higher rate of BRAF mutations and high microsatellite instability (MSI-H), both which have established prognostic importance, with MSI-H tumours shown to have a favourable prognosis, and BRAF a strong poor prognostic marker in non-MSI-H but not in MSI-H tumours[22,23,26,27]. In addition even within MSI-H tumours there are known differences in prognosis, with hereditary MSI-H colon cancers shown to have a better survival than sporadic cases[28]. It is important to note that these biomarkers are not uniformly distributed by stage, with MSI-H tumours associated with lower stage (21% in stage II vs 14% stage III and 4% stage IV), and BRAF mutant tumours more likely to occur at a higher stage[22,29,30]. Furthermore, previous studies have shown a differential effect of adjuvant chemotherapy in between molecular subtypes. There is a reduced benefit with fluorouracil based chemotherapy in MSI-H tumours, but preserved efficacy of oxaliplatin in MSI-H stage III colon cancer patients[31,32]. Although our study demonstrated a persistent effect of primary tumour location even when OS analysis was restricted to those patients who received adjuvant oxaliplatin doublet chemotherapy, it is important to note that fewer patients with RsCC received oxaliplatin as part of the adjuvant treatment.

Therefore, in the absence of both family history and molecular profiles in these population series, it is reasonable to hypothesise that some of the observed survival difference in stage II and III may be due to unequal distribution of these biomarkers. However, emerging evidence suggests that primary tumour location may be a clinical surrogate for further, yet unidentified, predictive biomarkers as highlighted by the recent data from the FIRE3 and CALGB/SWOG 80405 trials, which suggests a reduced benefit to anti-EGFR treatment in RsCC independent of currently identified biomarkers [33].

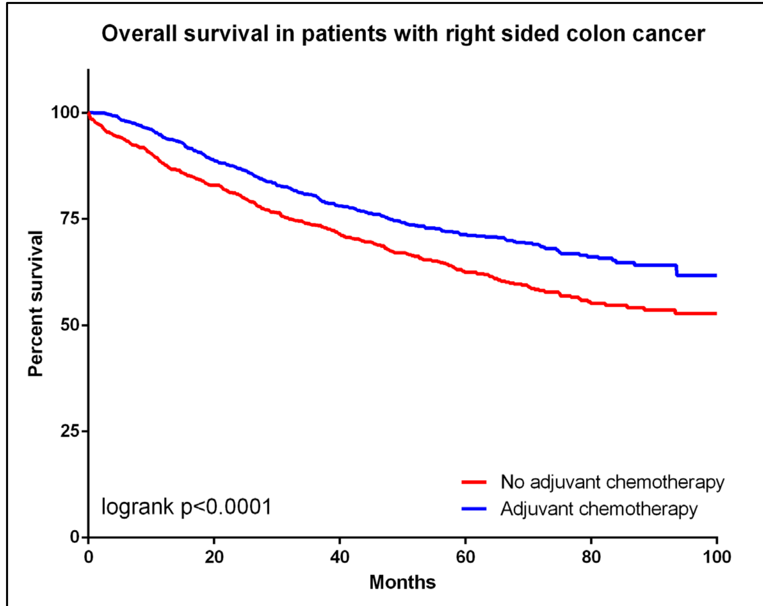
Recent retrospective analyses of these studies suggested that primary tumour site may impact the benefit of the EGFR monoclonal antibodies in patients with RAS wild type metastatic colorectal cancer. While the addition of EGFR antibodies to chemotherapy improved clinical outcomes in LsCC compared to the anti vascular endothelial growth factor (VEGF) antibody bevacizumab, the opposite was seen in LsCC. This intriguing result has prompted investigation for additional predictive biomarkers informed by primary tumour location. A limitation of our study is the lack of associated molecular data which is a potential source of unmeasured confounding to the results.

6.1.5 Conclusion

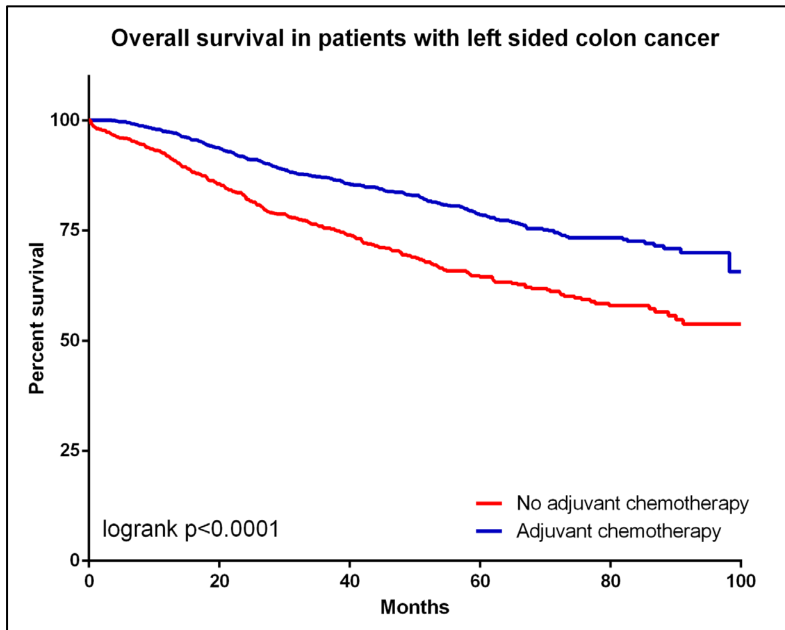
This population based study provides further evidence that primary tumour location is an important independent clinical prognostic factor in stage II and III colon cancer with immediate implications for clinical practice and trial design. This clinical biomarker is likely acting as a surrogate for as yet unidentified molecular factors. Further studies with associated tumour molecular profiles are required to clarify the underlying biological differences between RsCC and LsCC

6.1.6 Supplementary Material

Supplementary Figure 1: Effect of adjuvant chemotherapy on overall survival in patients with right sided colon cancer (n=2076).



Supplementary Figure 2: Effect of adjuvant chemotherapy on overall survival in patients with left sided colon cancer (n=2030).



Safety and Efficacy of Oxaliplatin Doublet Adjuvant chemotherapy in Elderly Patients with Stage III Colon Cancer

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Abstract

MicroAbstract: Due to poor representation in trials, the optimum adjuvant regimen for elderly patients with stage III colon cancer is uncertain. We employed data from a cancer registry to show a survival benefit with addition of oxaliplatin to fluoropyrimidine in patients ≥ 70 years. We note an increased rate of hospital admissions and early chemotherapy cessation in elderly patients on oxaliplatin.

Background: Colon cancer is common in the elderly, but due to under-representation in clinical trials, the benefit of standard therapies is uncertain in this age group. We aimed to clarify the efficacy and complications of adjuvant oxaliplatin and fluoropyrimidine chemotherapy for patients 70 years and older with stage III colon cancer.

Patients and Methods: All patients with stage III colon adenocarcinoma were identified from an Australian cancer registry (2006 – 2013). Multivariable Cox hazard regression was used to determine prognostic factors for all-cause mortality. Chemotherapy complications were quantified using discontinuation rates, hospital admissions, and mortality for 12 months after starting chemotherapy

Results: 2164 patients fulfilled our inclusion criteria including 1080 (49.9%) ≥70 years. Patients ≥70 years were less likely to receive adjuvant chemotherapy (60.7% vs 89.6%) or oxaliplatin doublet chemotherapy (18.8% vs 71.2%). Older patients receiving oxaliplatin were more likely to cease treatment early (18.7% vs 7.6%), and require hospital admission (67.0% vs 53.5%). The addition of oxaliplatin provided an overall survival benefit for patients <70 years (HR 0.44 95% CI 0.3 – 0.6, p<0.0001), and for patients ≥70 years (HR 0.64 95%CI 0.5 – 0.9, p=0.005).

Conclusions: Despite a modestly increased rate of hospital admission and early chemotherapy cessation, we demonstrate a persistent survival benefit for the addition of oxaliplatin to a fluoropyrimidine as adjuvant treatment for stage III colon cancer in elderly patients.

6.2.1 Introduction

Colon cancer is a common and lethal malignancy, with about 100,000 new cases diagnosed annually in the United States[34]. It is a disease related to aging, with almost 40% of colon cancer diagnosed in patients >75 years[35-37].

Surgical resection is the only curative treatment for locoregional disease, although many patients will develop disease recurrence due to micrometastases present at surgery. In resected stage III colon cancer, standard treatment includes adjuvant doublet chemotherapy with oxaliplatin and a fluoropyrimidine[38], following the results of several large phase III randomised control trials which showed a 30% reduction in disease recurrence and 22% reduction in risk of death with the addition of oxaliplatin to fluoropyrimidine alone[39-41].

Elderly patients appear to gain a similar benefit to fluoropyrimidine based adjuvant chemotherapy compared to younger patients[42]. However as only a minority of patients in clinical trials are older than 70 years, the efficacy and safety of adjuvant chemotherapy with an oxaliplatin doublet in elderly patients is unclear. For example, in the ACCENT database which includes individual patient data from 14500 participants in 18 fluoropyrimidine - based adjuvant trials, only 18% are older than 70 years[43].

Currently available trial data is conflicting. Subgroup analyses from the pivotal phase III MOSAIC and NSABO-07 trials show a survival benefit only in patients <70 years[39,40]. Similarly, there was no disease-free survival (DFS) or overall survival (OS) improvement with the addition of oxaliplatin in the 2575 patients ≥70 years in the ACCENT database[43]. In contrast however, pooled individual patient data from 904 patients ≥70 years from the NSABP C-08, XELOXA, X-ACT, and AVANT studies showed an attenuated, but statistically significant benefit to the addition of oxaliplatin, including those with comorbidities [44].

Similarly, retrospective patient series demonstrate contrasting results. While the largest series, drawn from multiple USA databases including the SEER database, found a statistically significant benefit to adjuvant oxaliplatin in elderly patients (70-74 years old) and those with comorbidities[45], this was less clear in patients >75[46] and was not seen in other, smaller studies[47,48].

As a consequence of these uncertainties, current guidelines recommend discussing incorporation of oxaliplatin with patients over 70 years based on individual circumstances, although fluoropyrimidine monotherapy is an appropriate choice for adjuvant therapy in the elderly[38,49].

The current study employs data from an Australian cancer registry to investigate the comparative effectiveness of the addition of oxaliplatin to fluoropyrimidine monotherapy as adjuvant treatment for stage III colon cancer in a "real world population" of patients older than 70.

6.2.2 Patients and Methods:

6.2.2.1 Patient Cohort

The New South Wales (NSW) clinical cancer registry contains demographic and clinical data for patients diagnosed or treated for cancer in NSW, covering approximately 30% of the Australian population. Data is collected from pathological laboratories, hospitals and oncology departments under mandatory notification of new cancer cases. We included all patients ≥18 years with colon cancer as per third edition of the International Classification of Diseases for Oncology (ICD-O-3)[13]. We identified 2220 patients with stage III colon cancer with complete files including adjuvant chemotherapy details. Fifty-six patients were excluded due to death within 30 days of surgery (n=23) or delay starting chemotherapy past 120 days (n=33) (final sample n=2164).

Date of death was obtained with linkage to the NSW registry of Births, Deaths and Marriages (BDM) by the Centre for Health Record Linkage (CHeReL) [14]. The censor date for survival data was 1st December 2014.

Comorbidity data and admissions during chemotherapy were obtained by CHeReL linkage of the clinical cancer registry data to the Admitted Patient Data Collection (APDC). The APDC contains all admitted patient services provided by New South Wales Public Hospitals, Public Multi-Purpose Services, Private Hospitals, and Private Day Procedures Centres. Comorbidities of each patient were quantified using the Charlson comorbidity index which predicts mortality from a range of 22 comorbid conditions[16]. ICD-10 codes were extracted from admissions prior to diagnosis, then translated into a Charlson comorbidity index (modified for cancer) using methods previously described[15,16].
For quantification of chemotherapy complications, all admissions for 12 months following initiation of chemotherapy were included apart from admissions for vascular implantation, chemotherapy, routine surgery follow-up, and dialysis (ICD-10 codes Z45.2, Z51, Z48.815, Z49 respectively). Admissions for febrile neutropenia were identified using neutropenia (ICD10 D70) with fever and/or sepsis (ICD10 R50.8, R50.9, A419) and/or infection (ICD10 Chapter A, B) as previously described for Australian patients[50].

Linkage and use of the data from the New South Wales (NSW) clinical cancer registry, the NSW registry of Births, Deaths and Marriages (BDM), Admitted Patient Data Collection (APDC) was

approved by the NSW Population & Health Services Research Ethics Committee (approval HREC/13/CIPHS/39).

6.2.2.2 Statistical analysis

Our primary outcome was all-cause mortality, on the basis of dates recorded in the cancer registry and BDM databases. Median values for OS and corresponding 95% CI were calculated using Kaplan-Meier methods. To determine the impact of age, two separate cox proportional hazard models were used to compare the effect of combination chemotherapy regimens on OS for patients ≥70 years and <70 years. This age cut-off was used for consistency with previous publications and international guidelines. The following variables were included in the multivariate model: age, sex, Charlson Comorbidity Index (CCI), TNM stage, primary tumor location (defined as right sided - caecum to transverse colon, or left sided - splenic flexure to rectosigmoid), year of diagnosis, grade, and adjuvant treatment.

Our secondary objectives were complications of adjuvant chemotherapy by age group, as measured by number and length of admissions for 12 months after starting treatment, and 12 month landmark mortality, and treatment discontinuation rate by chemotherapy regimen. The number of admissions was compared using ChiSq, and mean duration of each admission by the t-test. All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC).

6.2.3 Results:

6.2.3.1 Patient Characteristics (n=2164) and impact of age on receipt of chemotherapy

The characteristics of patients are summarised in Table 5. Approximately half the patients were ≥70 years (49.9%). Patients ≥70 were more likely to have right sided primary tumors (54.6% versus 42.7%) and poorly differentiated histology (24.3% versus 18.3%) but, despite these higher risk features, were less likely to receive adjuvant chemotherapy (60.7% versus 89.6%) or oxaliplatin doublet chemotherapy (18.8% vs 71.2%). Patients ≥80 years (n=371) were even less likely to receive chemotherapy; only 29.4% received adjuvant fluoropyrimidine monotherapy and 3.0% received oxaliplatin doublet. Increasing TNM stage was significantly associated with receipt of oxaliplatin chemotherapy patients <70 years (p=0.0006) but not in those ≥70 years (p = 0.08).

Table 5: Patient Characteristics

	All Patients n=2164 (%)	**Pts < 70 years n=1084 (50.1%)**	**Pts ≥ 70 years n=1080 (49.9%)**	***P* value**
TNM stage				
IIIa	272 (12.6)	141 (13.0)	131 (12.1)	0.81
IIIb	1284 (59.3)	638 (59.9)	646 (59.8)	
IIIc	608 (28.1)	305 (28.1)	303 (28.1)	
Charlson Comordibity Index				
0	1485 (68.6)	831 (76.7)	654 (60.6)	<0.0001
1-2	403 (18.6)	165 (15.2)	238 (22.1)	
3-4	195 (9.0)	63 (5.8)	132 (12.2)	
5 or more	81 (3.7)	25 (2.3)	56 (5.2)	
Primary Tumour Location				
Right	1053 (48.7)	463 (42.7)	590 (54.6)	<0.0001
Left	1111 (51.3)	621 (57.3)	490 (45.4)	
Age group				
<60	513 (23.7)	513 (47.3)		
60– 69	571 (26.4)	571 (52.7)		
70 – 79	709 (32.8)		709 (65.7)	
>=80	371 (17.1)		371 (34.3)	
Sex				
Male	1125 (52.0)	577 (53.2)	548 (50.8)	0.25
Female	1039 (48.0)	507 (46.8)	532 (49.3)	
Grade				
Well differentiated	220 (10.2)	112 (10.3)	108 (10.0)	0.0028
Moderately differentiated	1484 (68.6)	774 (71.4)	710 (65.7)	
Poorly differentiated	460 (21.3)	198 (18.3)	262 (24.3)	
Year Diagnosed				
2009 – 2009	1096 (50.7)	570 (52.6)	526 (48.7)	0.07
2010 – 2013	1068 (49.4)	514 (47.4)	554 (51.3)	
Adjuvant Chemotherapy				
None	538 (24.9)	113 (10.4)	425 (39.4)	<0.0001
Fluoropyrimidine monotherapy	651 (30.1)	199 (18.4)	452 (41.8)	
Oxaliplatin doublet	975 (45.0)	772 (71.2)	203 (18.8)	

Patients ≥70 years were more likely to have a higher CCI than younger patients (39.4% versus 23.3% with CCI >0). Increasing CCI was associated with decreased administration of adjuvant chemotherapy and oxaliplatin doublet treatments in all patients (p<0000.1), patients <70 years (p=0.04) and patients ≥70 years (p<0.0001).

While the majority of patients on fluoropyrimidine monotherapy received oral capecitabine rather than intravenous fluorouracil (83.9% versus 16.1%), only a minority patients treated with oxaliplatin doublet chemotherapy had oral capecitabine (CAPOX) rather than intravenous fluorouracil (FOLFOX) (13.7% versus 86.3%). There was a similar pattern of use patients ≥70 years and < 70 years.

6.2.3.2 Complications of chemotherapy

Chemotherapy complications were quantified with hospital admissions for 12 months following initiation of adjuvant chemotherapy, and 12 month landmark mortality.
In patients who received fluoropyrimidine monotherapy, there was no significant difference in proportion of patients <70 years admitted to hospital compared to those ≥70 years (49.7% versus 49.8%, p=0.59), or mean duration of admissions (5.92 days versus 5.59, p=0.66). In contrast, patients ≥70 years who received oxaliplatin were more likely to be admitted to hospital (67.0% vs 53.5%, p = 0.0006) and require multiple admissions (37.4% required ≥2 admissions vs 25.5%, p=0.0008) than younger patients on oxaliplatin. There was a non-significant trend to longer admissions (mean length of admission 6.1 days vs 4.8, p=0.09).

In patients ≥70 years, those treated with oxaliplatin were more likely to be admitted to hospital (67.0% vs 49.6%, p<0.0001) and require multiple admissions (37.4% ≥2 admissions vs 26.1%, p=0.003) than those on fluoropyrimidine monotherapy. While there was no significant difference in admissions for febrile neutropenia between age groups for patients on oxaliplatin (6.9% vs 4.7%, p=0.19), patients ≥ 70 years on oxaliplatin were more likely to be admitted for febrile neutropenia than those on fluoropyrimidine monotherapy (6.9% vs 1.8%, p=0.0008).

Patients ≥70 years were also less likely to complete adjuvant oxaliplatin doublet chemotherapy than those <70 years, defined as receiving <3 months of treatment (18.7% versus 7.6%, p<0.0001). There was no difference in completion rates between age groups for patients on fluoropyrimidine alone (p=0.33).

Patients ≥70 years who received adjuvant oxaliplatin doublet chemotherapy had a significantly poorer 12 month landmark OS than younger patients (5.9% vs 1.7%, p =0.0006). This difference between age groups was not seen in patients who received fluoropyrimidine monotherapy (8.6% vs 4.5%, p=0.06). Within patients ≥70 years, there was no significant difference in 12 month OS between those who received fluoropyrimidine monotherapy compared to oxaliplatin doublet (8.6% vs 5.9%, p =0.23).

6.2.3.3 Efficacy of adjuvant chemotherapy in the elderly

In all patients who received adjuvant chemotherapy (n=1626), oxaliplatin doublet chemotherapy improved OS compared to fluoropyrimidine alone (multivariate HR 0.54 95%CI 0.43 – 0.70, p<0.0001, Table 6, Figure 3). Increasing age, comorbidity score, TNM stage, poorly differentiated grade, and right sided primary tumor location were all significantly associated with poorer OS in both univariate and multivariate analyses. Use of capecitabine, rather than 5-fluorouracil, was not

significantly associated with OS in either the fluoropyrimidine monotherapy (p=0.82) or oxaliplatin doublet (p=0.48) treatment groups.

Table 6: Univariate and multivariate analyses for overall survival in all patients who received adjuvant chemotherapy (n=1626)

Characteristic		**Univariate HR (95%CI)**	***P* value**	**Multivariate (95%CI)**	***P* value**
Age	=<60	1	<0.0001	1	0.010
	60 – <70	1.0 (0.97 – 1.3)		0.97 (0.7 – 1.3)	
	70 – 80	2.3 (1.8 – 2.9)		1.4 (1.1 – 1.9)	
	>80	3.7 (3.0 – 4.8)		1.7 (1.1 – 2.4)	
Sex	Male	1	0.49	1	0.43
	Female	1.05 (0.91– 1.2)		0.9 (0.8 – 1.1)	
TNM	IIIa	1	<0.0001	1	<0.0001
	IIIb	1.5 (1.1 – 2.0)		2.3 (1.4 – 3.8)	
	IIIc	3.0 (2.2 – 4.0)		5.3 (3.2 – 8.6)	
Grade	Well/mod differentiated	1	<0.0001	1	<0.0001
	Poorly differentiated	1.8 (1.6 – 2.2)		1.6 (1.3 – 2.0)	
Primary tumor location	Right	1	<0.0001	1	0.0008
	Left	0.65 (0.56 – 0.75)		0.7 (0.57 – 0.86)	
Charlson Comorbidity index	0	1	<0.0001	1	0.0004
	1-3	1.3 (1.1 – 1.6)		0.8 (0.6 – 1.1)	
	4 or more	2.9 (2.3 – 3.7)		1.8 (1.22 – 2.6)	
Year Diagnosed	2009 – 2009	1	0.79	1	0.83
	2010 – 2013	0.98 (0.83– 1.2)		1.0 (0.9 – 1.1)	
Adjuvant Chemotherapy	Fluoropyrimidine only	1	<0.0001	1	<0.0001
	Oxaliplatin doublet	0.50 (0.41– 0.61)		0.54 (0.43–0.70)	

HR – Hazard Ratio; CI – confidence interval;

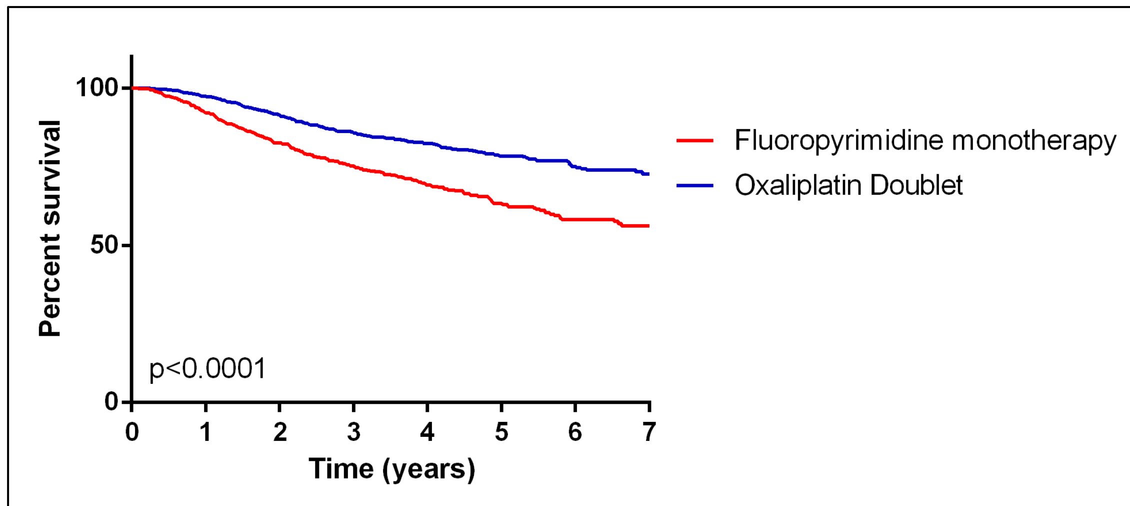

Figure 3: All-cause mortality by adjuvant chemotherapy regimen for all patients (n=1626)

When stratified by age, the addition of adjuvant oxaliplatin demonstrated an OS benefit in patients <70 years (HR 0.56 95% CI 0.41 – 0.77, p=0.0003) and ≥70 years (HR 0.72 95% CI 0.53 – 0.98, p = 0.037) which remained significant in multivariate analysis (Table 7 and Figure 4). Sex and year of diagnosis were not significant in univariate analysis and were therefore not included in the final model.

Table 7. Univariate and multivariate analyses for overall survival stratified by age

Characteristic		**Pts < 70 years (n=971)**			**Pts ≥ 70 years (n=655)**		
		Univariate HR (95% CI)	Multivariate (95% CI)	*P* value	Univariate HR (95% CI)	Multivariate	*P* value
TNM	IIIa	1	1	<0.0001	1	1	<0.0001
	IIIb	1.6 (0.9– 2.9)	1.7 (0.9 – 3.3)		1.5 (1.04 - 2.0)	3.0 (1.4 – 6.4)	
	IIIc	4.1 (2.3 – 7.1)	4.4 (2.3 – 8.2)		2.7 (1.9 – 3.9)	6.4 (3.0 – 13.9)	
Grade	Well/mod differentiated	1	1	0.0007	1	1	0.01
	Poorly differentiated	2.0 (1.5 – 2.7)	1.7 (1.3 – 2.5)		1.6 (1.4 – 2.0)	1.5 (1.1 – 2.0)	
Primary tumor location	Right	1	1	<0.0001	1	1	0.39
	Left	0.6 (0.5 – 0.8)	0.5 (0.4 – 0.7)		0.80 (0.6– 0.9)	0.9 (0.7 – 1.2)	
Charlson Comorbidity index	0	1	1	0.01	1	1	0.01
	1-3	0.8 (0.6 – 1.2)	0.6 (0.4 – 1.0)		1.2 (1.01– 1.5)	0.9 (0.6 – 1.2)	
	4 or more	2.4 (1.4 – 3.9)	1.8 (0.9 – 3.3)		2.4 (1.8 – 3.2)	1.9 (1.2 – 3.1)	
Adjuvant Chemotherapy Regimen	Fluoropyrimidine only	1	1	<0.0001	1	1	0.005
	Oxaliplatin doublet	0.6 (0.4– 0.8)	0.4 (0.3 – 0.6)		0.7 (0.5– 0.9)	0.6 (0.5 – 0.8)	

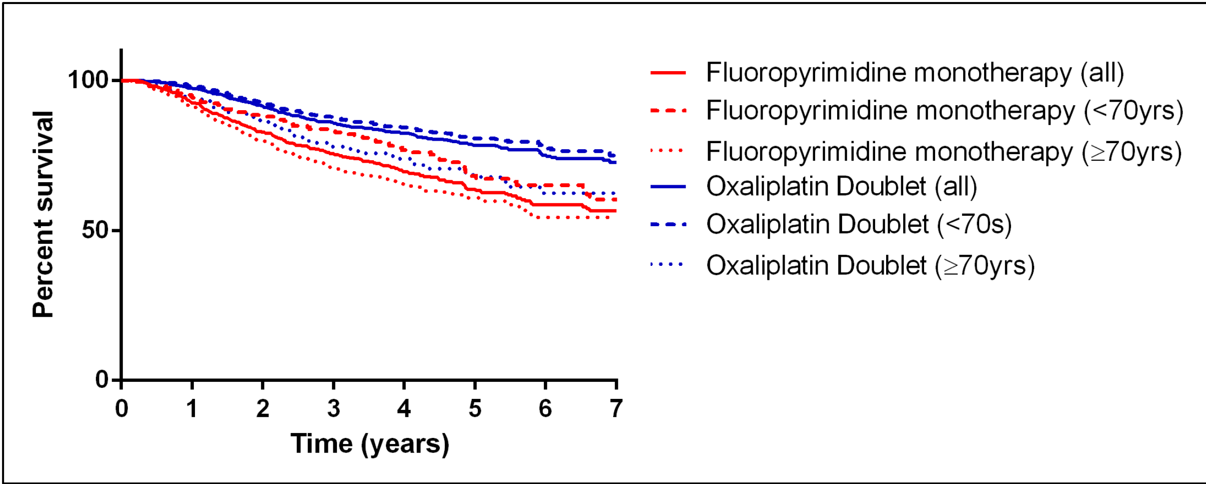

Figure 4: All cause mortality by adjuvant chemotherapy regimen stratified by patient age at diagnosis

Oxaliplatin doublet chemotherapy was associated with a preserved OS benefit in patients with significant comorbidity (patients with CCI≥2, univariate HR 0.40 95% CI 0.29 – 0.61, p<0.0001), including patients<70 (HR 0.38 95% CI 0.16 – 0.94, p=0.02), but not in patients ≥70 years (HR 0.67, p=0.28). Exploratory subgroup analysis demonstrated a diminishing OS benefit to oxaliplatin with increasing age, with the hazard ratio becoming approaching 1 (no benefit) for more elderly patients (Figure 5).

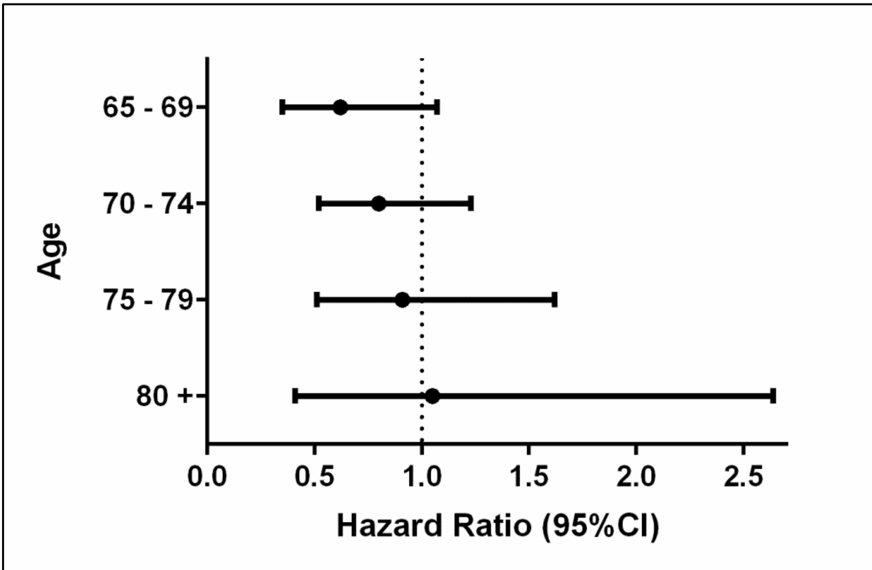

Figure 5: Unadjusted hazard ratio for overall survival benefit to oxaliplatin chemotherapy stratified by age. The circle on each bar represents the HR for that age group, and the bar shows the 95% confidence interval (CI).

6.2.4 Discussion

Colon cancer is more common in the elderly, since 67 is the median age at diagnosis, and almost 40% of patients are ≥75 years old[36,37]. Despite this, the pivotal phase III trials which demonstrated the improved OS with adjuvant oxaliplatin chemotherapy in stage III colon cancer included a only small minority of patients older than 70, and consequently were unable to show a benefit to oxaliplatin in this population[36,51]. As a result of this, Australian guidelines recommend fluoropyrimidine monotherapy as the most appropriate adjuvant chemotherapy regimen in elderly patients, although European Guidelines recommend a more individualised approach[52,53]

The principle finding of the current study is a statistically significant improved OS with adjuvant oxaliplatin doublet chemotherapy compared to fluoropyrimidine monotherapy in patients ≥70 years with stage III colon cancer (HR 0.72, p=0.037). This difference remained significant in multivariate analysis which included a comorbidity index (multivariate HR 0.64, p=0.005).

There is no consensus in the literature regarding the benefit of adjuvant oxaliplatin for elderly patients. Neither DFS nor OS was significantly improved in the small minority of elderly patients in the MOSAIC or NSABP-07 trials, or ACCENT database[39,40,43] In contrast, pooled individual patient data from four other randomised trials, which included comorbidities as a covariate, demonstrated improved DFS and OS with oxaliplatin [44]. Similarly, there are disparate results seen in "real world" patient series. Analysis of the SEER database showed a persistent benefit to adjuvant oxaliplatin in patients >70, although with inconsistent results in patients older than 75, and in those with significant comorbidities[45,46] . Other results from smaller retrospective series are conflicting[47,54]. One common criticism of all the above studies, as well as the current work, is the omission of an assessment for medical frailty, an important and distinct entity to comorbidity[55].

It is important to highlight that the elderly patients who received oxaliplatin chemotherapy in the current study are likely to represent a highly selected subgroup. This is supported by the observed high completion rate of adjuvant oxaliplatin doublet chemotherapy in patients ≥70 years, although we note that data regarding chemotherapy dosing, dose reductions, and delays, which may provide further insight, are not available. Consistent with other published series, we found increasing age was associated with decreased receipt of any adjuvant chemotherapy and oxaliplatin doublet chemotherapy, with only 18.8% of patients ≥70 years, and 3% ≥ 80 years, receiving oxaliplatin[46,47,56]. Similarly, increasing level of comorbidity, quantified by the CCI, was also associated with decreased receipt oxaliplatin (p<0.0001), with most of the elderly patients who received oxaliplatin (86.2%) having minimal comorbidities (CCI <2).

Despite presumed patient selection for oxaliplatin doublet therapy, hospital admissions were modestly increased in elderly patients compared to younger patients. Elderly patients who received oxaliplatin doublet chemotherapy more likely to be admitted to hospital, require multiple admissions to hospital, or require admissions for febrile neutropenia. These observations were not seen in patients receiving fluoropyrimidine monotherapy, consistent with current literature which shows increased toxicity in the elderly from doublet chemotherapy, but not fluoropyriminde monotherapy[42,44,47,57,58]. We acknowledge that the hospital admission data do not reflect all toxicity, as at least some complications are likely to have been managed out of hospital. It also important to highlight we did not find a significant difference in 12 month landmark OS between chemotherapy regimens in elderly patients. The decision to proceed with adjuvant chemotherapy in an elderly patient is complicated and depends on many patient health and social factors. Patients age, comorbidities, and perceived minimal benefit are the predominant reasons for withholding adjuvant chemotherapy in elderly patients[59]. While the average life expectancy of an otherwise healthy 70-year old male and female is approximately 8 years and 14 years, respectively[38], many elderly patients have significant comorbidities that could shorten survival. While we used the 70yrs age cut-off in our primary analysis for consistency with other publications, our exploratory subgroup analyses demonstrated, as expected, a diminishing benefit to adjuvant oxaliplatin with increasing age. Moreover, while the benefit for adjuvant oxaliplatin increases with time in younger patients, in older patients it decreases, so by three years after surgery the competing mortality risks eliminate the benefit of doublet adjuvant chemotherapy[43]. The recently presented data from the IDEA collaboration supports a risk adapted approach to duration of adjuvant chemotherapy, with a shorter duration of adjuvant chemotherapy in lower risk disease to reduce treatment associated toxicities[60]. These data, along with our study, support the role of individualised treatment approach, rather than strict age cut-offs, when determining the optimal adjuvant strategy for elderly patients.

There are limitations to the current study. Firstly, we analysed an observational database and acknowledge important unmeasured confounders and selection bias between treatment groups. Secondly, there was no data available regarding chemotherapy dosing, dose reductions, or treatment delays, for any patients. However while it is likely that most of the elderly patients received dose modifications to improve tolerability[61], we still found an OS benefit.

6.2.5 Conclusion

Our study demonstrates a survival benefit to adjuvant chemotherapy with an oxaliplatin doublet over fluoropyrimidine alone for patients ≥70 years with stage III colon cancer. However, we also found evidence of modestly increased hospital admission rates with doublet treatment. The potential for survival benefit must be weighed against the increased risk of toxicities in this population, as well as individual patient life-expectancies, based on co-morbidities and other factors.

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Chapter 7

Discussion

This chapter contains an overview of this thesis. The principle findings of each of the previous chapters are presented, followed by a discussion of the clinical and research implications, and future directions for research.

7.1 Summary of principle findings

7.1.1 *The urokinase plasminogen activation system, in particular uPAR, is a clinically relevant biomarker in all stages of gastroesophageal cancer*

Chapter 2 presented the first systematic review and meta-analysis of urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and plasminogen activator inhibitor-2 (PAI-2/SerpinB2) expression in primary oesophageal, gastro-oesophageal junction, and gastric adenocarcinomas. The meta-analysis of clinicopathological associations, overall survival (OS) and recurrence free survival (RFS) provided strong evidence that higher expression of uPA, uPAR or PAI-1 is significantly associated with high risk clinicopathological features and poorer prognosis. High uPA expression was associated with a shorter RFS (HR 1.90, p=0.01) and OS (HR 2.21, p<0.0001). High uPAR expression was associated with poorer OS (HR 2.21 p<0.0001). High PAI-1 expression was associated with shorter RFS (HR 1.96 p=0.03) and OS (HR 1.84, p<0.0001). There was no significant association between PAI-2 expression and OS, although data was limited. Thus uPA, uPAR and PAI-1 are proven to be novel and clinically relevant biomarkers in resected gastro-esophageal cancer and, as such, have the potential to be developed as prognostic and/or therapeutic targets.

In **Chapter 3** uPAR expression was shown to be significantly associated with expression of cancer stem cell (CSC) makers. Tumours expressing CD44 and ALDH1 were more likely to have high expression of uPAR (p = 0.02 and 0.03 respectively). These results agree with previous work in other tumours showing co-expression of CSC markers and uPAR, and provide further evidence demonstrating the importance of uPAR expression on CSCs. Strong similarities between the uPAR positive phenotype and CSCs were also noted, including invasive potential, drug resistance, and poorer prognosis.

These findings are extended in **Chapter 3 appendix,** where, for the first time, evidence is provided that expression of uPAR is an independent prognostic factor of metastatic gastroesophageal cancer. High expression of uPAR on cancer cells at the invasive edge of metastatic deposits was associated with a shorter OS in multivariate analysis (HR 1.5 95%CI 1.1 - 2.1, p<0.0001). These findings validate the use of uPAR as a prognostic biomarker across all stages of gastroesophageal cancer.

Despite the above results, the study detailed in **Chapter 4 appendix** shows that uPAR expression does not improve the standard CK+/CD45-/DAPI+ phenotype for EpCAM selected CTCs. Only a weak association between tumour tissue and CTC uPAR expression was found, and there was no association between % uPAR positive CTCs and tumour stage. Most importantly, % uPAR+ CTCs

did not have any significant association with OS. These negative results are likely attributable to the isolation method employed in the study rather than a true depiction of the role of uPAR expression on CTCs. The CTCs were isolated using EpCAM immunomagnetic separation, which restricted analyses to high EpCAM expressing CTCs only, thereby omitting CTCs which have downregulated EpCAM as part of EMT or due to an EMT/CSC phenotype.

7.1.1.1 Significance of findings and future directions

These results provide level 1 evidence for the uPA system as an independent prognostic factor in primary gastroesophageal cancer, a finding which has immediate clinical applicability.

In addition, these results provide an improved understanding of the molecular underpinnings of gastroesophageal cancers which is fundamental for the development of improved molecularly targeted therapeutics. There is an increasing interest in the uPA system, particularly uPAR, as both a biomarker and treatment target in solid tumours. Local and international research groups continue to develop agents directed at the uPA system with promising pre-clinical results [1-3]. The co-expression of uPAR and CSC markers provide a strong rationale for anti-uPAR therapies as a novel approach to target the CSC sub-populations. In addition, promising results from early clinical trials suggest radiolabelled uPAR as an improved imaging modality for cancer diagnosis, staging, and risk stratification [4].

Despite the results of the CTC study, there remains a compelling rationale for uPAR to be considered as a strong candidate marker to improve CTC selection, particularly for isolating EpCAM negative CTCs with the capacity to invade the extracellular matrix and establish metastasis. Future work should therefore employ alternative methods to isolate CTCs such as targeting EMT/CSC markers for isolation, physical properties or negative selection (alternative methods comprehensively reviewed by Alix-Panabieres et al [5]). It is important to note that the widespread expression of uPAR on activated leukocytes, as would be expected in a pro-inflammatory state such as advanced malignancy, may limit the specificity of positive selection of CTCs using uPAR [6].

7.1.2 Expression of the cancer stem cell marker CD44 is an independent prognostic factor in metastatic gastroesophageal cancer

Chapter 3 provided the first evidence that expression of CSC markers on metastatic disease is an independent prognostic biomarker in gastroesophageal cancer. This IHC study, using deposits of metastatic gastroesophageal cancer from 36 patients, found a significant association between OS and expression of CD44 (HR 2.9 95%CI 1.3 – 6.9, p=0.008) and ALDH1 (HR 2.4 95% CI 1.01 – 5.7, p=0.04) in univariate analysis. In multivariate analyses, after controlling for tumour grade, ECOG performance status, and treatment received, CD44 remained an independent predictor of poorer OS (HR 2.5 95%CI 1.1 – 6.2, p=0.04). The result for ALDH1 was not significant in multivariate analyses, but it is important to note the limitations of the small sample size.

There was no increased expression of CSC markers in tumours of patients who had received chemotherapy. Although this result appears contradictory to the well described chemoresistance of CSC, it is consistent with modern refinements to the CSC theory, which describe a fluid CSC population regulated by the tumour-cell environment, rather than a rigid hierarchical structure [7,8]. Following enrichment after chemotherapy, the CSCs reconstitute the differentiated bulk of the tumour mass. As all samples were taken at clinical progression, we posit there was sufficient time for the equilibrium between the CSC and bulk of tumour to be restored.

7.1.2.1 Significance of findings and future directions

These results provide the first evidence that CD44 CSC marker expression is an important biomarker in metastatic gastroesophageal cancer, and provide further support to the key role of CSCs in the molecular pathogenesis of gastroesophageal cancer. It is important to note the small sample size employed in the study, which necessitates further validation using a larger cohort in the future.

As addressed in Section 1.2.2, there is a growing evidence base to support the incorporation of CSC targeting in the treatment of cancer. Our results, which are the first to confirm and quantify CSC expression in metastatic gastroesophageal cancer, provide additional validity to the ongoing development of clinical trials of CSC directed treatment in metastatic cancers. We eagerly await the results of numerous trials for CSC targeted agents in gastroesophageal cancer (summarised by Bekaii-Saab *et al* [9]).

7.1.3 *Cryopreservation of CTCs is valid strategy for enumeration and biomarker detection in gastroesophageal cancer*

Chapter 4 details a robust protocol for the cryopreservation and thawing of the patient samples for CTC isolation and characterisation after long term storage. This study was conducted in two phases; firstly, the reliability of our protocol was confirmed with only a minor loss of CTCs observed between matched cryopreserved and fresh samples collected at the same blood draw. Secondly, a larger cohort of cryopreserved specimens was used to validate our method by demonstrating the prognostic association of CTC enumeration from cryopreserved specimens. In addition, the ability to extensively characterise CTCs isolated from cryopreserved specimens was shown by staining for a novel biomarker, uPAR, on the thawed CTCs.

7.1.3.1 Significance of findings and future directions

A persisting challenge to the field of circulating tumour cell (CTC) research has been the requirement for prompt analysis of samples at specialised centres. This has presented significant logistical challenges to researchers, compounded by the significant expertise, time and laboratory resources required for CTC analysis. Our results demonstrate the feasibility and validity of cryopreservation of CTCs, which has wide ranging and significant impacts on both research and clinical care. Cryopreservation has the potential to dramatically increase the number and range of studies using CTC analysis. It will assist with the wider incorporation of CTC collection and analysis in biobanking, retrospective studies, and large international clinical trials, by facilitating specimen storage, bulk transporting, and batch processing. It may also help to develop diagnostic settings that can service even remote patients with diagnostic CTC data potentially relevant for their disease management.

7.1.4 *Circulating tumour cell culture provides an accurate in vitro model for the study of metastasis*

Chapter 5 details the establishment and characterisation of two novel CTC cell lines from patients with gastroesophageal cancer. These two cell lines demonstrate distinct genotypic and phenotypic profiles providing a unique insight into disparate pathobiological mechanisms in metastatic gastroesophageal cancers. One of these cell lines (RFCTC), established from a patient with high grade neuroendocrine tumour, exhibited strong expression of neuroendocrine markers (chromogranin A+/synaptophysin+/CD56+) but no EpCAM expression and only weak cytokeratin staining. The other cell line (JICTC), derived from a patient with a distal gastric adenocarcinoma, displayed a strong CK+/EpCAM+/CD44+ phenotype. Both cell lines demonstrated rapid tumour growth in immunodeficient mice.

7.1.4.1 Significance of findings and future directions

These newly established CTC cell lines are highly significant for the following key reasons:

They are first CTC cell lines described in metastatic gastroesophageal cancer, and one of the few CTC cell lines described worldwide. Moreover, RFCTC is one of only several high grade gastrointestinal neuroendocrine cell lines. Both these cell lines provide a valuable addition to the limited worldwide resources to facilitate ongoing research into metastasis formation and high throughput drug testing.

The cell lines provide further evidence to support CTC culture as a feasible and clinically relevant laboratory technique. CTC culture continues to be characterised by modest success rates. This was consistent with our experience; 2/23 (8%) patient samples successfully developed long term CTC cultures. However, these CTC cultures were established without highly specialised laboratory equipment, and have been maintained without the need for highly defined culture media. Our results support the broad application of CTC culture as a laboratory technique, although further protocol refinements are needed to improve culture success rates.

Our results highlight the increasingly recognised limitations of EpCAM selection as an isolation method for CTCs, by demonstrating highly tumourigenic cell lines established form EpCAM+ and EpCAM- CTCs. Isolation techniques which do not include EpCAM- CTCs are likely to miss biologically relevant subpopulation of cells. The optimal technique for isolating and defining CTCs continues to be a rapidly evolving field.

As targeted treatments become more ubiquitous, there is an urgent need to develop improved methods

for personalising treatment through preclinical modelling. *Ex vivo* culturing of CTCs provide a critical tool to study cancer metastases, in addition to providing an ideal platform for the individualised preclinical testing for functional drug and radiotherapy testing. While other developing liquid biopsy techniques, such as ctDNA provide dynamic molecular data on tumour progression and resistance, CTC culture has the additional benefit of providing viable tumour cells for functional testing. The long term promise of *ex vivo* CTC culture is the development of a rapid preclinical "drug avatar" model for the optimisation of individual treatments, providing assistance for clinical decision making in real time. While low success rates and delays in confirming successful cultures are key ongoing challenges to this goal, our success provides assurance that this paradigm is achievable.

7.1.5 *Primary tumour location is an independent prognostic factor in locoregional colon cancer*

The prognostic role of primary tumour location in locoregional colon cancer remains a hotly debated and highly relevant topic. **Chapter 6.1** used multivariable cox hazard regression on a prospectively collected database, the New South Wales clinical cancer registry, to demonstrate that primary tumour location is an independent prognostic indicator in stage II (HR 0.85, p=0.02) and III (HR 1.13, p=0.032) colon cancer.

This study, using the most comprehensive population based database available in the literature, had several important distinguishing features from previously published work which reduce bias and validate the findings;

Firstly, by linking the cancer registry with a second prospectively collected database, the admitted patient database, a clinically validated measure of comorbidity – the "Charlson Comorbidity Index"- was able to be generated for each individual patient. This provides a much more detailed view of participant's comorbidities than any other published study, minimising the impact of selection bias in the results.

Secondly, by linkage to a third prospectively collected database, the births, deaths and marriage registry, an accurate date and cause of death was obtained for each patient, allowing a hazard regression models of both overall survival and cancer specific survival. The inclusion of cancer specific survival provided confirmation of our primary OS results.

Thirdly, the details of adjuvant chemotherapy, including regimen, was included for a subset of patients (including the majority of patients with stage III disease). Primary tumour location is known to be a significant factor on chemotherapy effect in colon cancer, and inclusion of the chemotherapy regimen reduced the confounding seen in most other reported series.

7.1.5.1 Significance of findings and future directions

The results contribute to a growing body of evidence demonstrating that primary tumour location is an important prognostic factor in colon cancer. Due to the strengths discussed above, this study provides the most robust population-level data to support primary tumour location as an independent prognostic factor in early stage colon cancer. Moreover, these results have immediate clinical applicability. The included study population was a modern cohort of Australian patients managed with currently employed treatments, and the results therefore can be directly applied to routine clinical care.

The observed difference in prognosis between LsCC and RsCC is postulated to be due to differences in tumour biology. While some differences in the molecular profile between LsCC and RsCC, such as BRAF mutations and microsatellite instability, are well described, ongoing research is urgently needed to further characterise and compare molecular profiles of LsCC and RsCC. Primary tumour location may be a clinical surrogate for further, yet unidentified, predictive biomarkers as highlighted by the recent data from the FIRE3 and CALGB/SWOG 80405 trials, which suggests a reduced benefit to anti-EGFR treatment in RsCC independent of currently identified biomarkers [10]. These results support the inclusion of primary tumour location as a stratification factor in clinical trials for all stages in colon cancer.

7.1.6 Adjuvant chemotherapy has a preserved OS benefit in the elderly; A comprehensive clinical assessment, rather than age alone, should be used when deciding adjuvant chemotherapy regimens in colon cancer.

Despite recent progress in immunotherapy and targeted agents, chemotherapy is the only adjuvant treatment shown to improve survival in stage III colon cancer. However, due to poor representation in clinical trials, the optimum adjuvant treatment is unknown in elderly patients, presenting a daily clinical dilemma to medical oncologists. **Chapter 6.2** provides a critical new source of evidence for this contentious issue. A large, prospectively collected, comprehensive, cancer registry dataset linked to governmental hospital data was used to quantify adjuvant chemotherapy rates and regimen choice for elderly patients. Multivariable cox hazard regression models demonstrated a persistent survival benefit to adjuvant oxaliplatin doublet chemotherapy for stage III colon cancer in the elderly, although at a cost of increased toxicity in this age group, demonstrated by an increase in chemotherapy discontinuation rates and hospital admissions.

7.1.6.1 Significance of findings and future directions

This study is the first population-level data series to incorporate comorbidities into multivariate models, and explore the toxicities associated with treatment using hospital admission data, providing a uniquely comprehensive analysis of benefits and risks of adjuvant oxaliplatin doublet chemotherapy in the elderly patient. The results support an individualised treatment approach, rather than strict age cut-offs, when determining the optimal adjuvant strategy for elderly patients.

These results remain limited by the observational design of the study, and the potential for unmeasured confounders and selection bias between treatment groups. The currently recruiting PRODIGE34 clinical trial, which randomises elderly patients to adjuvant oxaliplatin doublet or fluoropyrimidine monotherapy, aims to provide a definitive answer to this question [11].

7.2 Conclusions

Despite recent progress, gastrointestinal cancers remain highly lethal diseases and the predominate cause of cancer related deaths worldwide. Ongoing research is required to build on recent successes to improve the care and lives of patients with these cancers. Not only are new biomarkers and targets desperately needed, but also the optimisation of existing treatments to maximise benefits to patients. We continue to work with hope to shape a future where patients with early stage disease are cured, and the majority of patients with metastatic gastrointestinal cancer have long term survival and a good quality of life.

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Research Paper

The urokinase plasminogen activation system in gastroesophageal cancer: A systematic review and meta-analysis

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ABSTRACT

Background: The urokinase plasminogen activation (uPA) system is a crucial pathway for tumour invasion and establishment of metastasis. Although there is good evidence that uPA system expression is a clinically relevant biomarker in some solid tumours, its role in gastroesophageal cancer is uncertain.

Results: We identified 22 studies encompassing 1966 patients which fulfilled the inclusion criteria. uPA, uPAR, or PAI-1 expression is significantly associated with high risk clinicopathological features. High uPA expression is associated with a shorter RFS (HR 1.90 95% 1.16–3.11, $p = 0.01$) and OS (HR 2.21 95% CI 1.74–2.80, $p < 0.0001$). High uPAR expression is associated with poorer OS (HR 2.21 95%CI 1.82–2.69, $p < 0.0001$). High PAI-1 expression is associated with shorter RFS (HR 1.96 96% CI 1.07–3.58, $p = 0.03$) and OS (HR 1.84 95%CI 1.28–2.64, $p < 0.0001$). There was no significant association between PAI-2 expression and OS (HR 0.97 95%CI 0.48–1.94, $p < 0.92$) although data was limited.

Materials and Methods: We undertook a systematic review evaluating expression of uPA, urokinase plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and plasminogen activator inhibitor-2 (PAI-2/SerpinB2) on primary oesophageal, gastro-oesophageal junction, and gastric adenocarcinomas. We performed a meta-analysis of clinicopathological associations, overall survival (OS) and recurrence free survival (RFS).

Conclusions: We conclude that the uPA system is a clinically relevant biomarker in primary gastroesophageal cancer, with higher expression of uPA, uPAR and PAI-1 associated with higher risk disease and poorer prognosis. This also highlights the potential utility of the uPA system as a therapeutic target for improved treatment strategies.

INTRODUCTION

Gastroesophageal cancer is a common and lethal malignancy, marked by modest response to systemic therapies [1]. A deeper understanding of molecular events characterising carcinogenesis, invasion, progression and metastasis is central for the development of novel therapies.

The uPA system

A key process in the development and progression of cancer, including establishment of metastatic disease, is the invasion of malignant cells into normal tissue. The plasminogen activation system, particularly the urokinase-type plasminogen activator (uPA) system, is critical for tumour-associated proteolysis to breakdown extracellular matrix (ECM) and basement membranes barriers [2]. The uPA system has a defined role in tissue degradation and extravascular fibrinolysis, and is responsible for most of the activated plasminogen associated with cancer invasion and metastasis [2, 3] (Figure 1).

The uPA protein is secreted as a zymogen and activated on high affinity binding to its specific cell surface receptor uPAR. Once activated, uPA catalyses the activation of co-localised plasminogen to plasmin, which in turn directly degrades components of the ECM, and promotes further degradation and tissue remodelling by activating pro-metalloproteinases and by releasing, thus activating, latent growth factors from the ECM [4].

The uPA receptor (uPAR) is anchored to the plasma membrane, localising the uPA system to the cell surface [5]. High expression of uPAR on the invasive front of tumours facilitates invasion and other roles in cellular migration and angiogenesis [6]. uPAR expression may be a suitable marker for the onset of invasion of both gastro-intestinal and breast cancer as it is expressed only on invasive carcinomas, not premalignant states such as Barrett's oesophagus [7].

Urokinase-type plasminogen activator is efficiently inhibited by two subtypes of serpin (serine proteinase inhibitor) family members, plasminogen activator inhibitor-1 (PAI-1/SerpinE1) and –2 (PAI-2 /SerpinB2). Both form a covalent complex with uPA/uPAR leading to internalisation of the entire complex [8] . Although believed to have a physiological role as an inhibitor of the uPA system, PAI-1 has a paradoxical protumourgenic role, increasing tumour invasion and angiogenesis, and correlated with poor prognosis [9]. The role of PAI-2 in cancer is less clear. Although both PAIs mediate uPA/uPAR endocytosis, the uPA-PAI-2 complex interacts with endocytosis receptors with different binding kinetics to those of uPA:PAI-1 and without stimulating intracellular signalling events over and above that of uPA binding to uPAR [10].

While the uPA system is expressed on both cancer cells and the supporting stroma, higher expression is seen on tumour cells, and is postulated that the tumour cell specific uPA/uPAR explains the aggressive biology exhibited by these cancers, and is more relevant for prognostic outcomes [11–14]. Expression of the uPA system has been shown to be an important prognostic marker in a variety of cancers including breast cancer

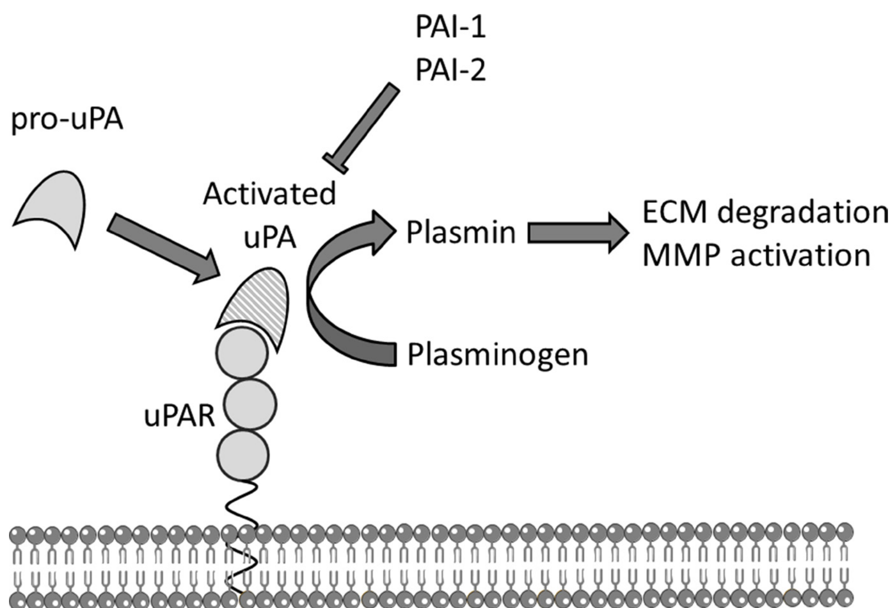

Figure 1: The uPA system. Schematic representation of the urokinase plasminogen activation (uPA) system. The membrane bound urokinase receptor (uPAR) binds circulating inactive pro-uPA, facilitating the activation of pro-uPA to uPA which subsequently converts co-localised plasminogen to plasmin that can directly degrade components of the extracellular matrix (ECM) and activate pro-matrix metalloproteases (MMP) to further break down ECM. Plasminogen activator inhibitors 1 or 2 (PAI-1, PAI-2) are efficient endogenous inhibitors of uPA.

[15], lung cancer [16], and colorectal cancer [17], with the combination of uPA and PAI-1 expression recommended to be incorporated into routine clinical care of node negative breast cancer [18].

In this study we aim to perform a comprehensive systematic review of expression of the uPA system encompassing uPA, uPAR, PAI-1, and PAI-2 in primary, resectable gastro-oesophageal cancer, and undertake meta-analyses of prognostic outcomes (recurrence free survival and overall survival), and association with relevant clinicopathological variables. To the best of our knowledge, this is the first meta-analysis to examine and compare the expression of these key components of uPA system in primary gastro-oesophageal cancer.

RESULTS

Included studies

The trial flow is provided in Figure 2. We identified 267 reports matching criteria for inclusion in the study, of which 109 were selected for abstract review, and 60 subsequently for full text review. Forty one studies (including 2689 patients) fulfilled criteria for inclusion in the systematic review, with 22 studies (1966 patients) providing sufficient data for inclusion in the formal quantitative meta-analysis: 19 studies were excluded for the following reasons: 12 studies did not examine prognostic or clinicopathological associations, 3 reports were matched case control studies, and 4 studies reported insufficient published data to derive a HR.

The characteristics of the included studies are summarized in Supplementary Table 1. Eighteen studies evaluated uPA system expression in gastric cancer (1732 patients), one study included oesophageal, junctional and gastric cancers (39 patients), and two studies examined oesophageal cancer only (105 patients). Expression of the uPA system was assessed using immunohistochemistry (IHC, 12 studies, 1273 patients), enzyme-linked immunosorbent assay (ELISA, 5 studies, 344 patients), reverse transcription polymerase chain reaction (RT-PCR, 3 studies, 153 patients), or in-situ hybridisation (ISH, one study, 105 patients).

Hazard ratios directly extracted for 3 studies [7, 11, 22]. The multivariate HR was used when univariate value was not provided [22]. When only subgroup outcome data (tumour core or peripheral zone) were available, the results for peripheral "invasion" zone were used [7, 11]. Hazard ratios were estimated for the remaining studies using published data. 4 studies reported a "non-statistically significant OS" result for uPA system expression, but did not publish sufficient data for inclusion in meta-analysis [23–26].

Bias risk

The risk of bias summary is summarized in Figure 3. Only 4 studies [22, 27–29] were deemed low risk in all bias domains. Fourteen studies did not clearly define the study population [7, 12, 13, 30–40] and 11 studies did not report completeness of followup [7, 12, 13, 30–33, 36, 38, 39, 41]. Most studies adequately reported method of measurement of the uPA system, although 5 studies did not report whether there was a second independent reviewer or blinding to clinical information [13, 35, 39, 40, 42]. The followup protocol was underreported in 14 studies [7, 11–13, 30–36, 38–40], although this is unlikely to bias the results for overall survival analyses. Most studies did not report details of the surgical, medical, or radiation treatments received by patients, and were Urokinase plasminogen activator (uPA).

Urokinase plasminogen activator (uPA)

uPA expression rates

Expression of uPA was evaluated in 13 studies (1254 patients). The mean expression of uPA was 52.8%, but had a large range (from 23% to 91%). There was no significant difference in mean expression for IHC (60.7%) and ELISA (45.6%) ($p = 0.10$).

uPA and clinicopathological associations

uPA expression is significantly associated with poorer clinicopathological features in resected gastroesophageal cancer including: Advanced T stage (T3/4 vs T1/2) (OR 2.79 95% CI 1.80–4.32, $p < 0.0001$), nodal metastases (OR 2.30 95% CI 1.63–3.51, $p < 0.0001$), liver metastases (OR 6.77 95% CI 2.70–16.96, $p < 0.0001$), peritoneal metastases(OR 2.09 95% CI 1.29–3.36, $p = 0.003$), lymphatic invasion (OR 2.28 95% CI 1.31–3.97, $p = 0.0003$), and vascular invasion (OR = 2.43 95% CI 1.53–3.86, $p = 0.0002$) (5 studies, 522 patients, Supplementary Figure 1). There is no significant association with histology (poorly differentiated vs well differentiated).

uPA expression and prognosis

uPA expression was significantly associated with a worse RFS (3 studies, 467 participants, HR 1.90 95% 1.16–3.11, $p = 0.01$) (see Supplementary Figure 2). There was no significant difference in RFS seen between studies using IHC (HR 1.77) or ELISA (HR 2.36) to assess uPA expression (test for subgroup differences $Chi^2 = 0.37$, $p = 0.54$).

uPA expression is significantly associated with poorer OS (12 studies, 1094 participants, HR 2.21 95% CI 1.74–2.80, $p < 0.0001$) (see Figure 4). There was no significant difference in OS between studies which used IHC (HR 1.94) or ELISA (HR = 2.99) to assess uPA expression ($p = 0.38$). Sensitivity analysis showed similar results when analysis was restricted to gastric cancer only (HR 2.07, $p < 0.00001$).

Urokinase plasminogen activator receptor (uPAR)

uPAR expression rates

Twelve studies (1127 patients) evaluated uPAR expression, with mean uPAR expression of 56.8% (range

14–90%), with similar mean expressions seen in IHC (56.8%) and ELISA/RT-PCR (56.7%).

uPAR expression and clinicopathological associations

uPAR expression on primary resected gastroesophageal cancer is significantly associated with poorer clinicopathological features including: advanced TMN stage (stage III/IV vs I/II, OR 3.41 91% CI 1.55–7.53, $p = 0.002$), advanced T stage (OR 2.33 95% CI 1.53 to 3.56, $p < 0.0001$), nodal metastases (OR 2.52 95% CI 1.70–3.72, $p < 0.0001$), liver metastases (OR 2.53 95% CI 1.25–5.13, $p = 0.010$), peritoneal metastases (OR 3.15 95% CI 1.87–5.28, $p < 0.0001$), lymphatic invasion (OR 2.82 95% CI 1.74–4.59, $p < 0.0001$) and vascular invasion (OR 3.85 95% CI 2.53–5.88, $p < 0.0001$) (six studies, 589 patients, Supplementary Figure 3). There is no significant association seen with histology ($p = 0.6$).

uPAR expression and prognosis

Only one study provided data for uPAR expression and RFS [42], showing a shorter RFS with uPAR expression (203 patients, HR 2.69, $p = 0.03$).

uPAR expression is associated with poorer OS (11 studies, 1036 patients, HR 2.19 95% CI 1.80–2.66, $p < 0.0001$) (Figure 5). There was no significant difference in OS seen between studies which used IHC (HR 2.13), ISH (HR 2.34), ELISA (HR 2.19), or RT-PCR (2.66) to assess uPAR expression ($p = 0.96$).

Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 expression rate

Twelve studies (1031 patients) examined PAI-1 expression. Mean PAI-1 expression was 53.3%, with no

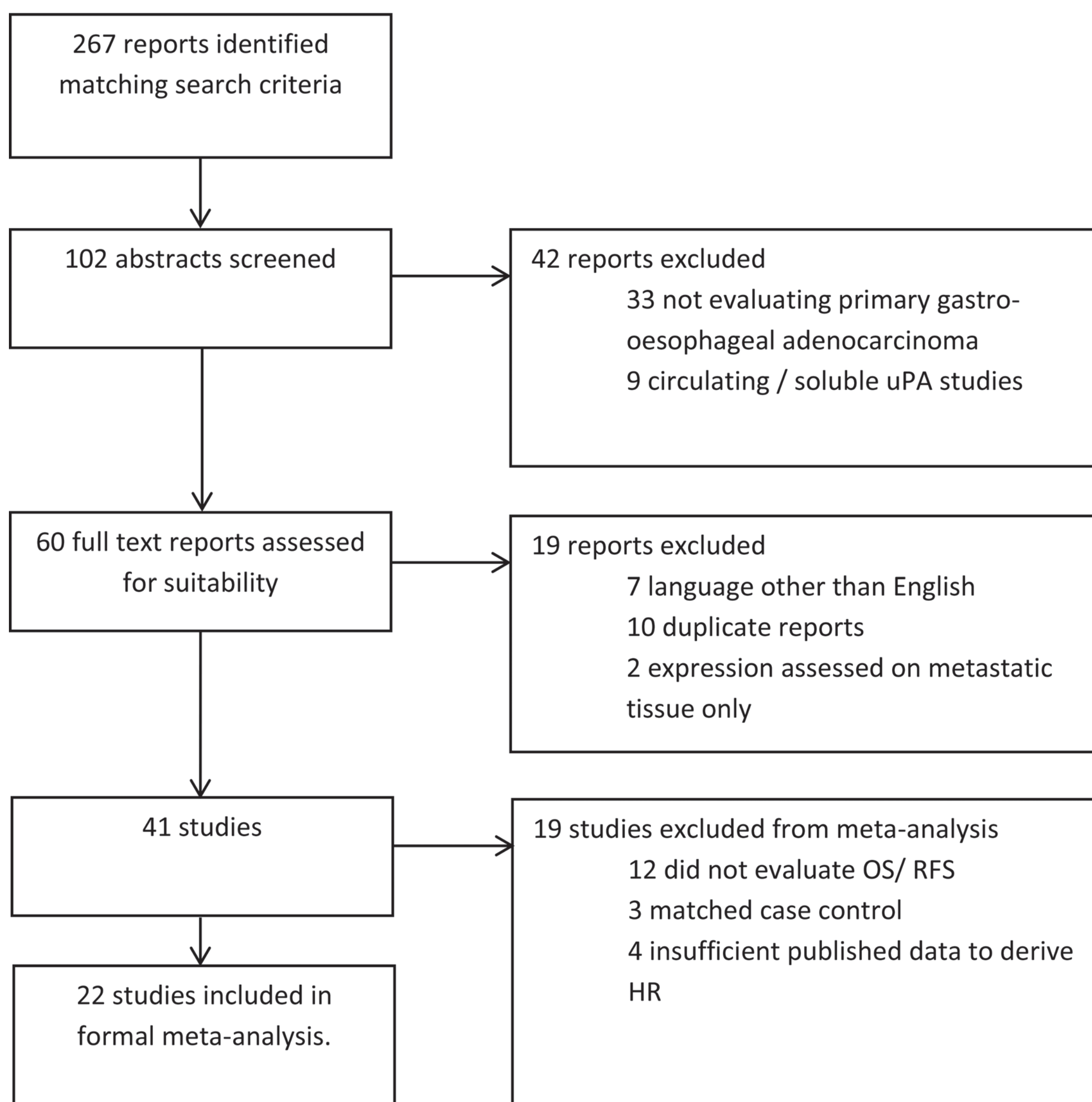

Figure 2: Study selection flow diagram. HR –hazard ratio; OS–overall survival; RFS–recurrence free survival.

statically significant difference in expression between IHC (61.8%) and RT-PCR/ELISA (44.7%) ($p = 0.1$).

PAI-1 expression and clinicopathological variables

PAI-1 expression on primary resected gastroesophageal cancer is significantly associated with poorer clinicopathological features including: advanced T stage (OR 2.59 95% CI 1.61 to 4.18, $p < 0.0001$), nodal metastases (OR 2.03 95% CI 1.27–3.22, $p < 0.003$), lymphatic invasion (OR 2.09 95% CI 1.31–3.34, $p < 0.004$) and vascular invasion (OR 1.90 95% CI 1.20–3.03, $p < 0.007$) (three studies, 317 patients, Supplementary Figure 4). There was no significant association of PAI-1 expression with presence of liver metastases (OR 0.52, $p = 0.18$), peritoneal metastases (OR 1.38, $p = 0.31$), or histology (OR 0.93, $p = 0.74$).

PAI-1 expression and prognosis

PAI-1 expression is associated with shorter RFS (3 studies, 467 patients, HR 1.96 96% CI 1.07–3.58, $p = 0.03$) (Supplementary Figure 5). There was no significant difference in RFS between studies which used IHC or ELISA to detect PAI-1 expression ($p = 0.86$)

PAI-1 expression is significantly associated with a shorter OS (10 studies, 839 participants, HR 1.84 95%CI 1.28–2.64, $p < 0.0001$, Figure 6). Pre-specified subgroup analysis showed a significant difference between studies which assessed PAI-1 expression using IHC (HR 1.20, $p = 0.47$) and ELISA (HR 2.94, $p < 0.0001$) or RT-PCR (HR 2.83, $p < 0.0001$) ($p = 0.02$).

Plasminogen activator inhibitor-2 (PAI-2)

PAI-2 expression rate

Two studies (145 participants) assessed PAI-2 expression (all using IHC) (refer to Supplementary Table 1). Mean expression was 57.5%.

PAI-2 expression and clinicopathological variables

There were no studies with sufficient data analyzing PAI-2 expression and clinicopathological variables for inclusion in the meta-analysis.

PAI-2 expression and prognosis

No studies published data on PAI-2 expression and RFS. There was no significant association of PAI-2 expression and OS (2 studies, 145 participants, HR 0.97 95%CI 0.48–1.94, $p < 0.92$, Supplementary Figure 6).

Publication bias

Examination of the funnel plots for the OS analysis for uPA, uPAR and PAI-1 showed asymmetrical plots for all analyses, suggesting absence of smaller negative trials (example plot for uPA provided in Supplementary Figure 7).

	Bias Domain					
Study	Study Participation	Study Attrition	Prognostic factor Measurement	Outcome Measurement	Study Confounding	Statistical analysis and reporting
Allott 2012	Orange	Orange	Green	Orange	Orange	Green
Alpizar Alpizar 2012	Green	Green	Green	Orange	Orange	Orange
Bayer 2006	Green	Green	Green	Green	Green	Green
Cho 1997	Green	Green	Green	Green	Green	Green
Ganesh 1996	Green	Green	Green	Green	Green	Green
Heiss 1995	Green	Green	Orange	Green	Green	Green
Ito 1996	Orange	Orange	Green	Orange	Orange	Orange
Kaneko 2003	Orange	Orange	Green	Orange	Orange	Green
Kawasaki 1998	Orange	Orange	Green	Orange	Orange	Green
Laerum 2012	Orange	Orange	Green	Orange	Orange	Green
Lee 2004	Orange	Orange	Green	Orange	Orange	Green
Luebke 2006	Green	Orange	Green	Green	Orange	Green
Maeda 1996	Orange	Green	Orange	Orange	Orange	Green
Murata 1998	Orange	Green	Green	Orange	Orange	Green
Nekarda 1994	Green	Green	Green	Green	Green	Green
Nekarda 1998	Green	Green	Green	Green	Green	Green
Okusa 1999	Orange	Orange	Green	Orange	Orange	Green
Plebani 1997	Orange	Green	Green	Green	Green	Green
Sakakibara 2006	Orange	Orange	Green	Orange	Orange	Green
Taniguchi 1998	Orange	Orange	Orange	Orange	Orange	Green
Yonemura 1997	Orange	Orange	Orange	Orange	Orange	Green
Zhang 2006	Orange	Green	Orange	Orange	Orange	Green

Figure 3: Risk of bias summary. For each bias domain: green = "low risk" means that sufficient data was available to allow assessment of quality and fulfilled criteria for each domain, and accordingly is deemed low risk of bias. Orange = "unclear risk" means that insufficient data was presented to adequately assess the quality of the domain and accordingly the study has potentially high risk of bias. There were no studies deemed high risk of bias.

DISCUSSION

This meta-analysis confirms the clinical utility of the uPA system as a biomarker in resected gastro-oesophageal adenocarcinoma.

There is good evidence that high expression of uPA, uPAR, and PAI-1 is associated with most high risk clinicopathological features, including advanced T stage, presence of nodal and distant metastases, and lymphovascular invasion, in primary gastro-oesophageal adenocarcinoma. This supports the central role of the uPA system in tumour invasion and metastasis. In contrast, there was no significant association of expression found with poorly differentiated histology, consistent with previously published work which shows that epithelial cell uPA system expression is higher in malignant than benign tissue, but decreases as tumour becomes more poorly differentiated, with a corresponding increase in stromal expression [43].

We also demonstrated that uPA, uPAR, and PAI-1 expression is associated with poorer prognosis in resected gastro-oesophageal cancer, with both a shorter RFS and OS in tumours which expressed these markers. However this result should be interpreted with caution due to the following important limitations in our study.

Firstly, only four of the included studies were deemed low risk for all bias domains as assessed by the QUIPS tool. In particular, most studies did not report the treatments patients received which is an important potential source of confounding for RFS and OS analyses. Additionally, tumours with higher risk clinicopathologic features could reasonably be expected to be more likely to have received neoadjuvant treatment prior to surgery, which may in turn have impacted on the expression of the uPA system. Despite this, it should be noted that similar results were seen in studies deemed low and high risk of study confounding, and heterogeneity was low in both the uPA and uPAR OS meta-analyses ($I^2 = 31\%$ and 0% respectively, see Figures 4 and 5).

Secondly, there is evidence of underreporting of non-significant results. This is demonstrated by both the funnel plot, as well the selective reporting of only statistically positive findings from included studies. This important bias will cause an overestimation of the effect of expression.

Thirdly, as demonstrated above, tumours that expressed uPA, uPAR and PAI-1 had higher risk features,

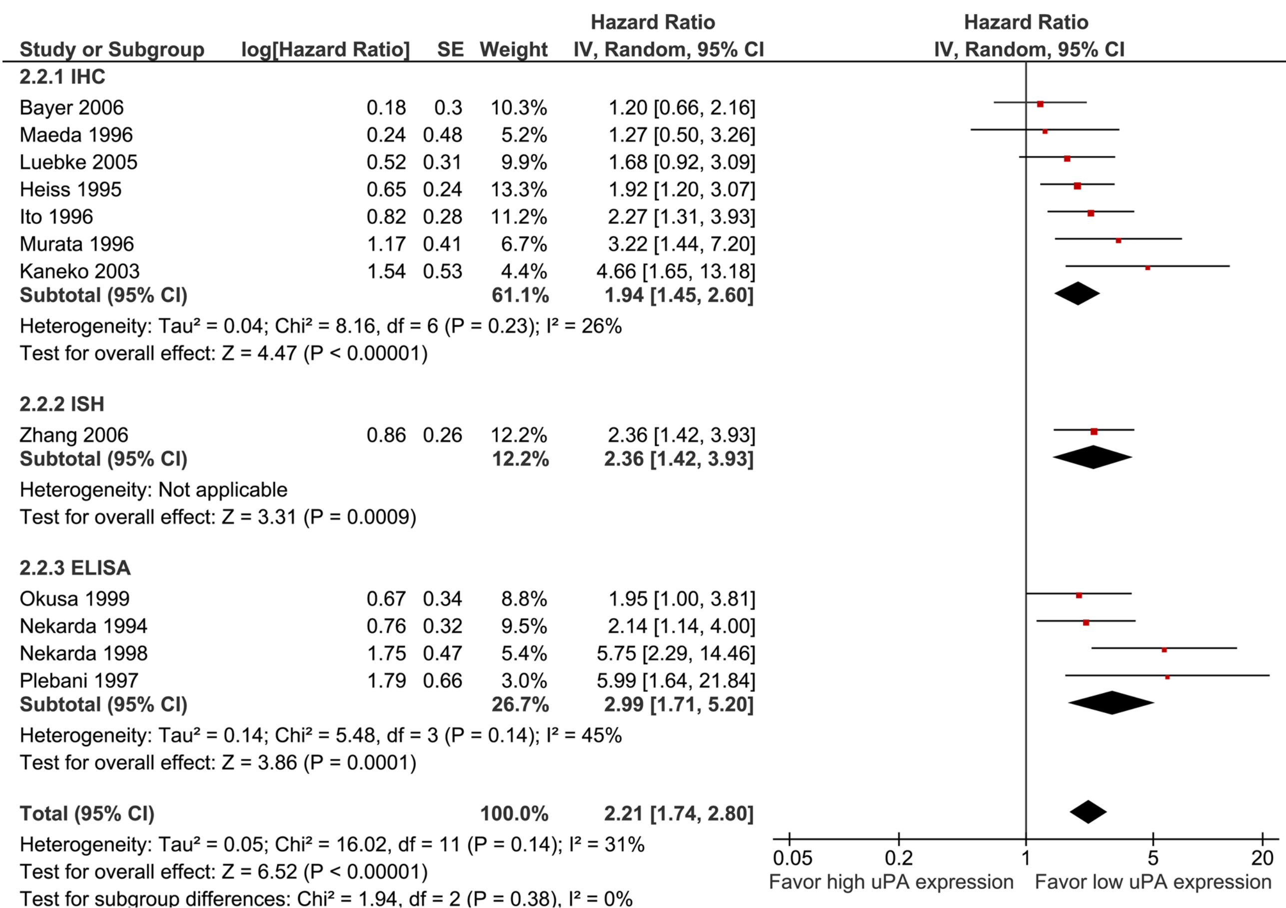

Figure 4: Pooled estimate of hazard ratio (HR) for uPA expression and overall survival (OS). Pooled estimate of hazard ratio (HR) for overall survival. The square on each bar represents the HR for an individual trial, and the bar shows the 95% confidence interval (CI). The diamond represents a pooled estimate with the centre of the diamond giving the HR estimate, and the extremes of the diamond representing the 95% CI. 24.

and would be expected to recur or progress sooner than tumours that did not. The apparent difference in prognostic outcomes may be due to unequal baseline characteristics of the included participants.

We did not show a significant difference in the prognostic outcomes between studies which used a tumour cell specific technique (e.g. IHC) compared to whole tissue lysates (e.g. RT-PCR, ELISA) for uPA and uPAR. This is consistent with other studies which have shown correlation between IHC score and median ELISA value, and supports the cancer cells as a major source of uPA and uPAR expression in the tumour tissue [44].

In contrast, there was a significant different in the expression methodology subgroups in the analysis for PAI-1 and OS ($p = 0.02$), with a non-significant outcome seen in studies using IHC (HR 1.20, $p = 0.47$), compared to significant results with ELISA (HR 2.94, $p < 0.0001$) and RT-PCR (HR 2.83, $p = 0.0007$). This highlights the importance of the stromal production of PAI-1 within the tumour microenvironment [9], as only methods that took into account both stromal and tumour PAI-1 showed statistically significant prognostic outcomes. It has been postulated that in contrast to uPAR, fibroblasts and endothelial cells provide the major source of PAI-1 within the tumour tissue [45]. It is possible that the PAI-1 detected on the tumour cells by IHC may be explained by internalization and accumulation of stromal produced uPA-PAI-1 complexes mediated by tumour uPAR [46]. No IHC studies examined the association between stromal PAI-1 expression and prognostic outcomes in gastro-oesophageal cancer.

All IHC study results used in the meta-analysis were restricted to tumour cell expression only. Similar to other cancers, uPA system expression was highest at the invasive front of the tumour [7, 11, 12, 31]. Only four

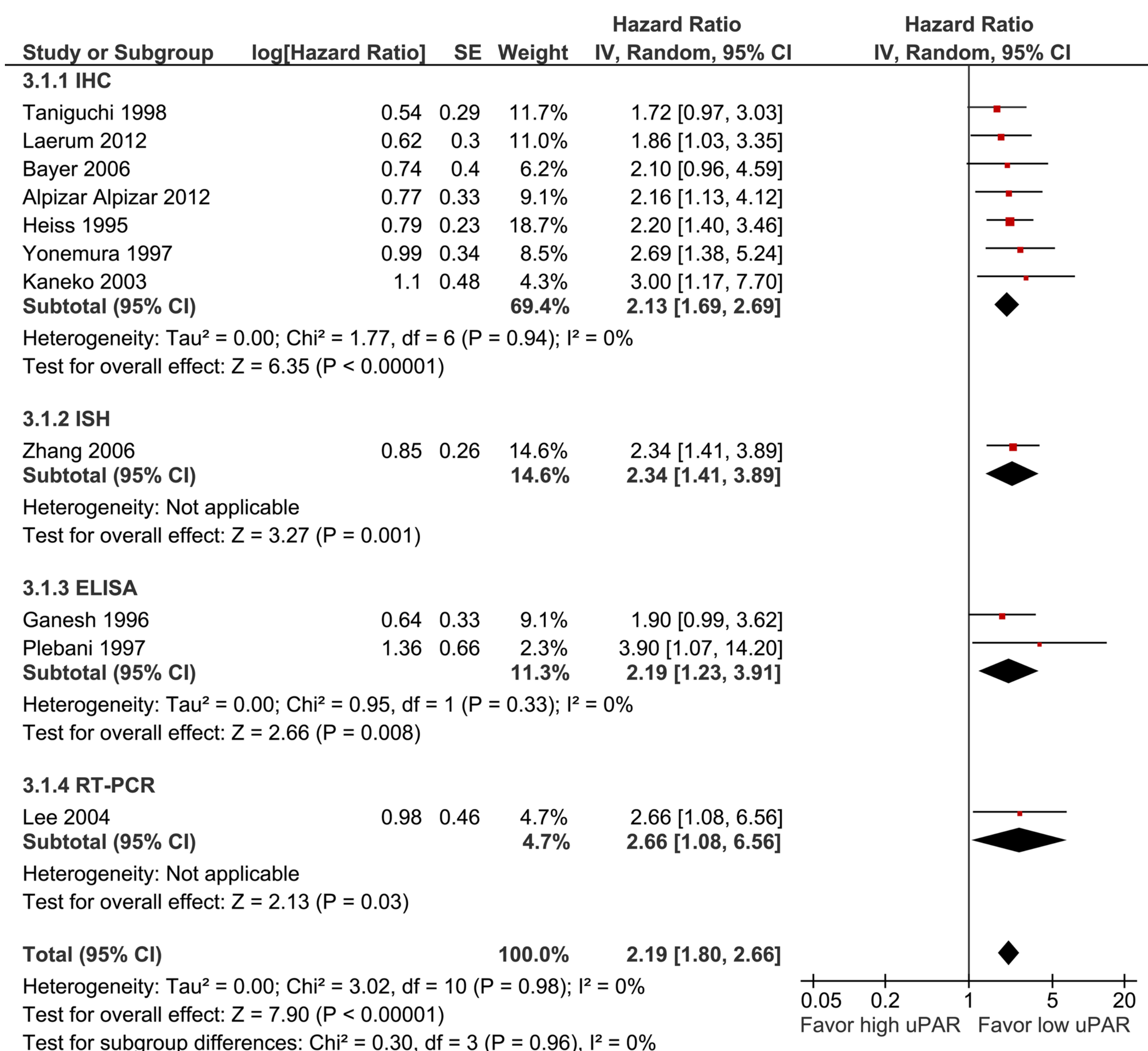

Figure 5: Pooled estimate of hazard ratio (HR) for uPAR expression and overall survival (OS).

studies reported stromal expression of the uPA system [7, 11, 12, 42]. Results were conflicting, with only one study showing a significant association of OS with macrophage uPAR expression on the invading zone at the periphery of the tumour [7]. In colorectal cancer, high uPAR expression on macrophages in the tumour core, rather than the periphery, is an independent predictor of poor prognosis [47]. These studies suggest an important supporting role of the tumour associated macrophages within the tumour microenvironment. The contrasting pattern of high uPAR expression (core versus peripheral) may be due to differing phenotypes of the subpopulations of tumour preventing (M1 macrophages) and tumour promoting (M2 macrophages) macrophages within the heterogeneous tumour bulk [48] . Further work is required to elucidate the biology of the stroma in gastrointestinal cancers.

We were unable to show any significant associations with PAI-2 expression with either clinicopathological features or prognostic outcomes, as available data was much more limited. Similarly only 3 studies examined oesophageal cancer, which limits applicability of our results to this subgroup. Sensitivity analysis did not show a different result when oesophageal cancer was excluded from analysis.

In conclusion, expression of the uPA system is a clinically relevant biomarker in gastroesophageal cancer. There is good evidence to support the association of uPA, uPAR, and PAI-1 expression and high risk clinicopathological features. While we found a statistically significant association between uPAR, uPAR and PAI-1 expression and poorer prognosis, our results are tempered by methodical limitations discussed above. Our findings also highlight the potential utility of the uPA system as a therapeutic target for improved treatment strategies.

MATERIALS AND METHODS

Methods are reported according to Preferred Reporting for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [19].

Study eligibility/selection criteria

We included all studies which examined the following components of the urokinase plasminogen activation system uPA, uPAR, PAI-1 or PAI-2, in resected primary esophageal, gastroesophageal junction, or

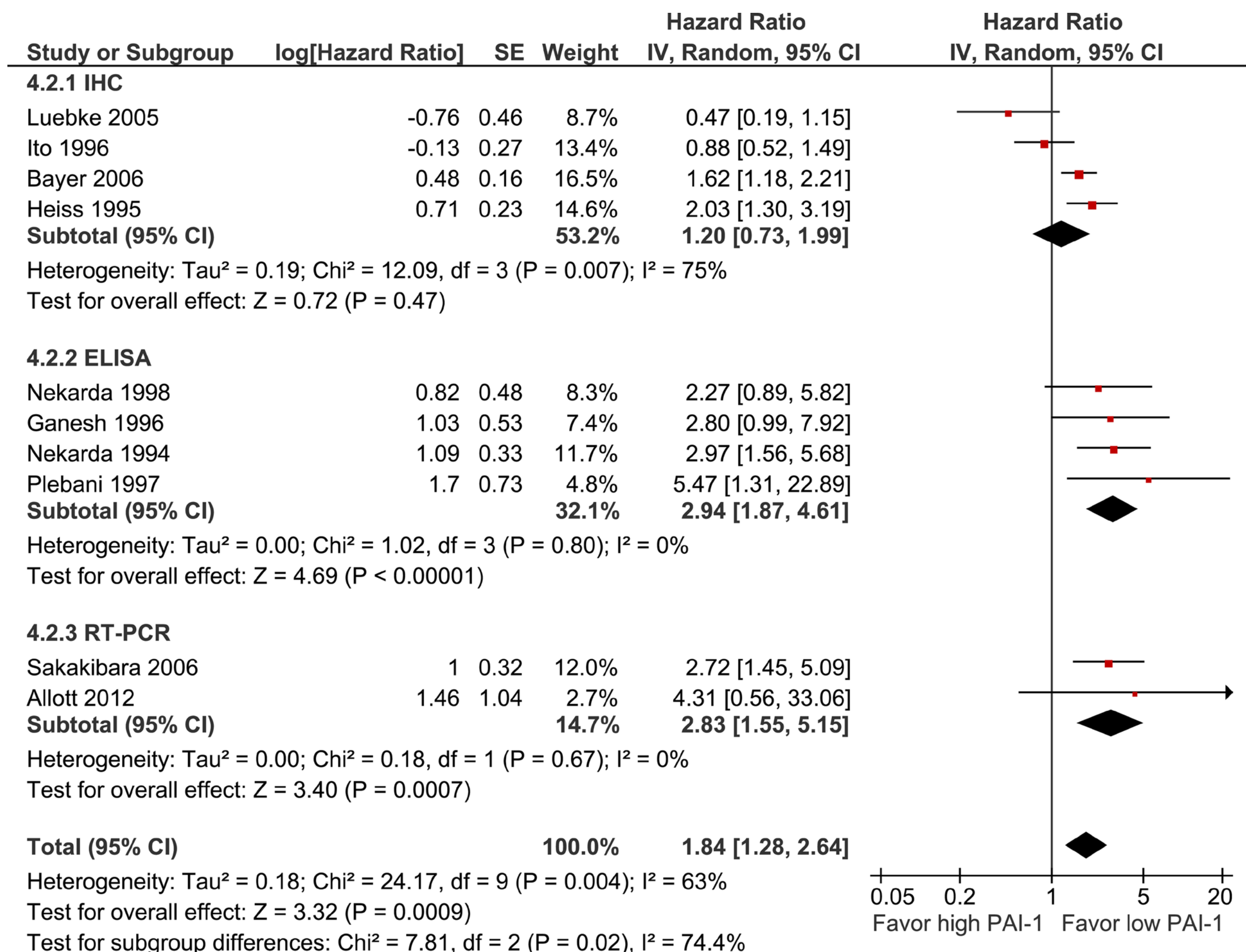

Figure 6: Pooled estimate of hazard ratio (HR) for PAI-1 expression and overall survival (OS).

gastric adenocarcinomas. Other tumour pathologies were excluded. A ll methods of assessing expression, including reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), in-situ hybridization (ISH), and immunohistochemistry (IHC) were included. For inclusion in the meta-analysis, studies were required to report the association of the following outcomes with uPA system expression: overall survival (OS), recurrence-free survival (RFS), or clinicopathological variables.

Two authors (DB, JC) independently performed the search and screened the studies. The primary outcome was OS; secondary outcomes were RFS, and correlation of clinicopathological variables with uPA system expression.

Study search strategy

We searched the following databases in February 2015 for all trials fulfilling the above criteria: Medline (1950–present); EMBASE (1966–present); Cochrane Central Register of Controlled Trials, and Cochrane Database of Systematic Reviews; PubMed.

To maximize sensitivity the following search terms were used: Stomach Neoplasms (MESH) OR Esophageal neoplasms (MESH) OR Gastrointestinal neoplasms (MESH) OR Gastric cancer.mp OR Gastric carcinoma.mp OR esophageal cancer.mp OR oesophageal cancer.mp OR gastroesophageal cancer.mp AND Receptors, urokinase plasminogen activator (MESH) OR Urokinase-type plasminogen activator (MESH) OR Plasminogen activator inhibitor 1 (MESH) OR Plasminogen activator inhibitor.mp OR PAI-1.mp OR PAI-2.mp OR Urokinase* plasminogen.mp OR uPA*.mp. Reference lists of included studies and review articles were hand searched. The search was restricted to studies published in English.

Data collection

Study data was independently collected by two authors (DB, JC) using standardized electronic data collection forms. The following was collected for each study: patient number, primary tumour location (gastric/oesophageal/COJ), cancer stage, treatment received by patient; uPA components assessed (uPA, uPAR, PAI-1, PAI-2) and method, patient followup; outcomes (OS or RFS), clinicopathological correlations (including TMN stage, tumour grade, lymphatic invasion, vascular invasion). For studies which used IHC, expression analysis was restricted to tumour cells only (stromal expression was not included in the meta-analysis).

Assessment of bias within studies

All studies included in the meta-analyses were assessed for bias using the Quality In Prognosis Studies (QUIPS) tool which assesses for potential sources of bias in six domains namely: study participation; study attrition and loss to followup; prognostic factor measurement; outcome measurement; study confounding; and statistical analysis and reporting [20].

Statistical analysis

We extracted the hazard ratio (HR) and their 95% confidence intervals (CI) for time-to-event outcomes including RFS and OS. If both univariate and multivariate HR were published the univariate results were preferentially used. Where no HR was provided in published data, it was estimated from available results or Kaplan-Meier survival curves using previously described methods [21].

HRs were synthesized using the generic inverse variance method and a random effect model using RevMan5.1 analysis software. Statistical heterogeneity was assessed using the I^2 statistic. We performed pre-specified subgroup analysis for overall survival for: primary location (gastric or oesophageal), cancer cell specific expression (using IHC) compared to whole cell lysis (using RT-PCR/ELISA).

Clinicopathological associations were summarized using odds ratios (OR) derived from published results. This analysis was limited to studies using IHC, as other methods presented expression results as means, rather than percentage of patients expressing. Expression rates were described with mean and range, and compared using the student's *t*-test.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Basic Study

Cryopreservation for delayed circulating tumor cell isolation is a valid strategy for prognostic association of circulating tumor cells in gastroesophageal cancer

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Abstract

AIM

To demonstrate the feasibility of cryopreservation of peripheral blood mononuclear cells (PBMCs) for prognostic circulating tumor cell (CTC) detection in gastroesophageal cancer.

METHODS

Using 7.5 mL blood samples collected in EDTA tubes from patients with gastroesopheagal adenocarcinoma, CTCs were isolated by epithelial cell adhesion molecule based immunomagnetic capture using the IsoFlux platform. Paired specimens taken during the same blood draw (n = 15) were used to compare number of CTCs isolated from fresh and cryopreserved PBMCs. Blood samples were processed within 24 h to recover the PBMC fraction, with PBMCs used for fresh analysis immediately processed for CTC isolation. Cryopreservation of PBMCs lasted from 2 wk to 25.2 mo (median 14.6 mo). CTCs isolated from pre-treatment cryopreserved PBMCs (n = 43) were examined for associations with clinicopathological variables and survival outcomes.

RESULTS

While there was a significant trend to a decrease in CTC numbers associated with cryopreserved specimens (mean number of CTCs 34.4 *vs* 51.5, P = 0.04), this was predominately in samples with a total CTC count of > 50, with low CTC count samples less affected (P = 0.06). There was no significant association between the duration of cryopreservation and number of CTCs. In cryopreserved PBMCs from patient samples prior to treatment, a high CTC count (> 17) was associated with poorer overall survival (OS) (n = 43, HR = 4.4, 95%CI: 1.7-11.7, P = 0.0013). In multivariate analysis, after controlling for sex, age, stage, ECOG performance status, and primary tumor location, a high CTC count remained significantly associated with a poorer OS (HR = 3.7, 95%CI: 1.2-12.4, P = 0.03).

CONCLUSION

PBMC cryopreservation for delayed CTC isolation is a valid strategy to assist with sample collection, transporting and processing.

Key words: Cryopreservation; Circulating tumor cells; Liquid biopsy; Gastroesophageal cancer; Gastric cancer

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Core tip: This study demonstrates a novel and robust protocol for the cryopreservation and thawing of patient blood samples, demonstrating reliable circulating tumor cell isolation and characterisation after the long term storage of patient samples. Using the largest patient cohort reported to date, we validated our method by confirming the independent prognostic association of circulating tumor cell (CTC) enumeration from cryopreserved peripheral blood mononuclear cells. Cryopreservation may assist with the wider incorporation of CTC collection and analysis in biobanking, retrospective studies, and large international clinical trials, by facilitating specimen storage, bulk transporting, and batch processing.

Brungs D, Lynch D, Luk AW, Minaei E, Ranson M, Aghmesheh M, Vine KL, Carolan M, Jaber M, de Souza P, Becker TM. Cryopreservation for delayed circulating tumor cell isolation is a valid strategy for prognostic association of circulating tumor cells in gastroesophageal cancer. *World J Gastroenterol* 2018; 24(7): 810-818 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i7/810.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i7.810

INTRODUCTION

Circulating tumor cell (CTC) analysis continues to be a rapidly developing field in oncology, offering a promising tool to both prognosticate and guide managements for patients[1]. Despite recent advancements in the field, one persisting challenge to the widespread adoption of CTC analysis for translational clinical trials or routine clinical care is the limited time frame considered best for blood processing and CTC isolation. Usually fresh blood is processed for CTCs within 24 h after blood draw, requiring prompt transfer to specialised centres for CTC isolation and analysis, which offers significant logistical challenges[2]. To overcome this issue, some studies use blood collection tubes that contain fixatives. Fixation of blood samples can allow CTC processing delayed by several days which has proven very useful for some CTC analyses[3,4]. However, fixatives may interfere with down-stream molecular analyses that require isolation of nucleic acids[5]. An alternative is the use of cryopreservation protocols for peripheral blood mononuclear cells (PBMCs) to allow delayed CTC isolation from these cells followed by CTC analysis. Cryopreservation should overcome fixation related analysis limitations and allow far more flexible time frames for batched CTC processing. However, a defined, robust cryopreservation protocol that is proven to enable analysis of the same or at least a relevant proportion of CTCs to that found in fresh samples, needs to be adopted and confirmation is needed whether cryopreserved CTCs can still predict disease outcome.

The advantage of cryopreservation of PBMCs is that it requires only minimal local processing, possible in most diagnostic settings, as well as feasible cryostorage and frozen transport of PBMC samples.

While there are a large number of approaches used to isolate and identify circulating tumor cells (recently

reviewed by van der Toom *et al*[6]), the best established and most widely used is the CellSearch™ system (Menarini-Silicon Biosystems), which uses positive immunomagnetic isolation of epithelial cell adhesion molecule (EpCAM, an epithelial cell marker) expressing cells followed by cytokeratin (CK), CD45, and DAPI staining[2]. The CTCs are then identified with automated immunofluorescence microscopy, defined by an EpCAM/CK/DAPI positive and CD45 negative phenotype. Cell Search CTC counts have shown to be prognostic in large patient series in a variety of cancers[7-9], including gastroesophageal cancer[10-12], but the instrument offers limited sensitivity in resectable gastroesophageal cancer, with CTCs detected in less than 15% of patients[10,13]. The IsoFlux system (Fluxion) uses a similar definition of CTCs to CellSearch (EpCAM/CK/DAPI positive, CD45 negative phenotype), but has shown a greater sensitivity for CTC detection[14-16]. This platform uses EpCAM targeted immunomagnetic isolation of CTCs within a microfluidic setting, improving isolation of CTCs with lower EpCAM expression, minimising leukocyte contamination, and allowing downstream applications including staining, enumeration, or sequencing, as shown for fresh blood samples[16].

Here, we use a viable method of PBMC cryopreservation that allows subsequent isolation and immunocytochemical analysis of CTCs. We demonstrate the feasibility of PBMC cryopreservation for delayed CTC isolation using paired cryopreserved and freshly processed blood samples drawn at the same time from patients with gastroesophageal adenocarcinoma. Importantly, we also provide data confirming that cryopreserved CTCs remain clinically applicable as a circulating prognostic marker for overall survival (OS).

MATERIALS AND METHODS

Patient population

Blood samples were collected from patients with histologically confirmed distal oesophageal, gastroesophageal junction, or gastric adenocarcinomas treated at Wollongong Hospital, Australia. Blood samples were collected in 7.5 mL EDTA Vacutainer tubes (Sarstedt AG & Co.) and maintained at room temperature until processing.

In the initial cohort (Cohort 1) to confirm the feasibility of cryopreservation, 15 patients with gastroesophageal carcinomas had 2 specimens taken during the one blood draw, one processed within 24 h ("fresh" specimen), and one cryopreserved with delayed CTC isolation and analysis ("cryopreserved" specimen). Pretreatment blood samples were cryopreserved from a second, larger cohort of patients for correlation with clinical outcomes (Cohort 2). The study was approved by South Western Sydney Local Health District Human Research Ethics Committee (Project Number 15/072). A written informed consent was obtained from each participant before sample collection.

Sample preparation

Blood samples were processed within 24 h to recover the PBMC fraction using 50 mL SepMate tubes and Lymphoprep according to manufacturer's instructions (Stemcell Technologies, Vancouver, BC, Canada).

PBMCs used for fresh analysis were resuspended in Isoflux Binding Buffer and immediately processed for CTC isolation (see below).

PBMCs for cryopreservation were well resuspended in 1 mL of diluted plasma (the supernatant of the PBMC preparation from the matching patient) with the addition of 7.5% final DMSO, and stored at -80 ℃ until further processing. Cryopreserved samples were thawed according to the protocol from Fluxion Biosciences, San Francisco, California, United States[17]. In brief, warmed (37 ℃) thawing buffer, consisting of RPMI 1640 with 10% Fetal Bovine Serum (FBS, Bovogen Biologicals, Australia) and 50 Unit/mL Benzonase (Sigma-Aldrich, Germany), was added to thawed samples, washed once in thawing buffer, and resuspended in IsoFlux Binding Buffer with 5% FBS.

Circulating tumor cell isolation, staining, and imaging

As per the Fluxion protocol, immunomagnetic beads preconjugated with anti-EpCAM antibodies (CTC Enrichment Kit; Fluxion Biosciences Inc) were added to PBMCs suspended in IsoFlux Binding Buffer, and incubated for 90 min at 4 ℃ with passive mixing on a rotator. Samples were then loaded into the sample well of the microfluidic cartridge and underwent immunomagnetic isolation of CTCs with the IsoFlux using the standard protocol (Fluxion Biosciences Inc).

Recovered CTCs were blocked with a final concentration of 1.2 μg/μL mouse IgG in binding buffer (Jackson ImmunoResearch, Baltimore, PA, United States) for 30 min, washed and fixed in fixing solution (Fluxion Biosciences Inc). The CTCs were then blocked in 10% FBS in binding buffer for 15 min, then underwent immunofluorence staining for anti-CD45 antibody conjugated to Alexa Fluor 647 (Biolegend, Clone HI30). The CTCs were also stained for urokinase plasminogen activator receptor (uPAR, CD87), a key receptor in the plasminogen activator system and clinically relevant biomarker in primary gastroesophageal cancer[18], using anti-uPAR antibody conjugated to AF594 (ThermoFischer, Clone R4). After permeabilization with 0.1% Triton X-100, cells were probed with anti-cytokeratin antibody conjugated to FITC (Sigma-Aldrich, Clone PCK-26). CTCs were finally stained with Hoechst and mounted using Isoflux mounting media to 24-well glass bottom plates (MoBioTec, Goettingen, Germany) for imaging.

Imaging was performed with an inverted epifluorescence microscope (Leica DMi8, Leica Microsystems Pty Ltd) using the Leica Application Suite. Cells were considered CTCs if they were CK positive, CD45

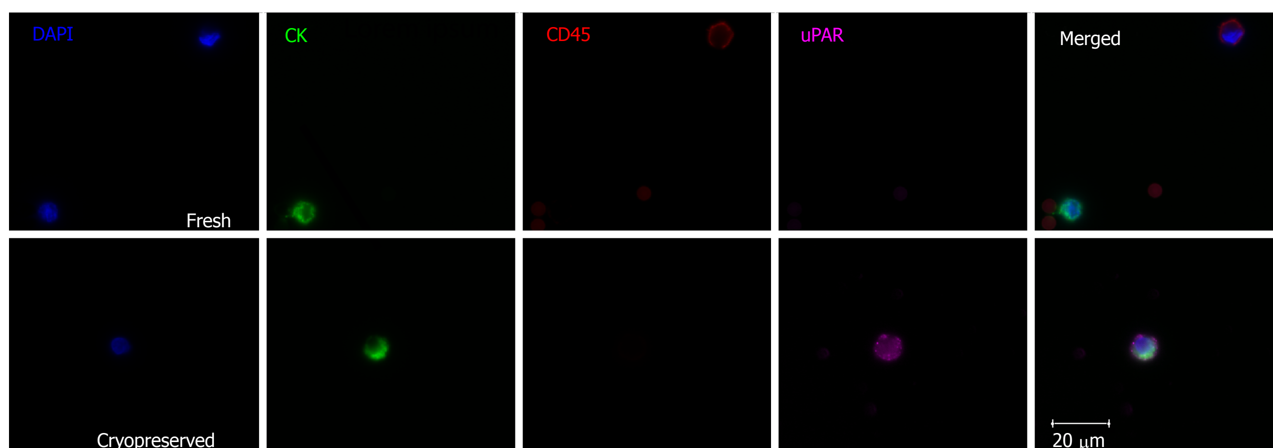

Figure 1 Representative images of circulating tumor cell isolation from fresh and cryopreserved samples demonstrating preservation of leukocyte and circulating tumor cell morphology. The fresh sample demonstrates a nucleated CK+/CD45- CTC which is uPAR negative, as well as a CK-/CD45+ leukocyte. The cryopreserved sample shows a uPAR positive CTC. CTC: Circulating tumor cell.

negative, nucleated and morphologically intact. The proportion of uPAR positive CTCs was recorded.

Statistical analysis

The CTC recovery from matched cryopreserved and fresh samples were compared with the paired *t*-test. Correlation between cryopreservation time and CTC number was described with a Pearson correlation coefficient, and the Fisher exact test and *t*-test were used to compare the status of CTCs with categorical clinicopathologic factors.

For survival analyses, in the absence of established cut-offs for prognostic CTC numbers, the median CTC count (17) was used as the discriminator between high and low CTC counts. Survival analyses are conducted using Kaplan-Meier methods, with median survival reported. Unadjusted and multivariable Cox proportional hazards regression analyses were used to estimate the association between CTC counts and survival, and to calculate corresponding hazard ratios (HRs) and 95% confidence intervals (CIs). The following variables were included in the multivariate model: age, sex, ECOG, TNM stage, primary tumor location, and CTC count. All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC, United States).

RESULTS

Matched fresh and cryopreserved specimens (cohort 1)

Matching parallel blood samples, collected from 15 gastroesophageal cancer patients (10 patients had blood taken prior to treatment, 5 patients were already on treatment), that had either been cryopreserved before CTC processing or were processed fresh, were compared. Cryopreservation of PBMCs lasted from 2 wk to 25.2 mo (median 14.6 mo). There was no significant correlation between cryopreservation time and CTC number (Pearson r -0.25, P = 0.09). CTCs isolated from cryopreserved samples appeared morphologically similar to fresh samples (Figure 1). There was a significant difference between CTC numbers isolated from the cryopreserved samples compared to fresh samples (mean number of CTCs 34.4 cryopreserved *vs* 51.5 fresh, P = 0.04, Figure 2), however this difference was predominately attributable to a larger fall in CTC numbers in samples with very high CTC counts (> 50 CTCs in the fresh specimen). There was no significant difference in CTC count between cryopreserved and fresh samples for specimens with CTC count less than 50 (n = 11 patients, mean number of CTCs 10.7 *vs* 16.3, P = 0.06). Thus CTC loss by cryopreservation in patient samples with low CTC counts appears relatively minor (mean proportion of CTCs lost in cryopreserved samples = 23.95%).

Cryopreserved circulating tumor cell and clinical outcomes (cohort 2)

A larger cohort of 43 gastroesophageal cancer patients (cohort 2) was analyzed to validate whether detectable CTC counts post cryopreservation correlated to disease outcomes. All patient samples were taken prior to treatment commencement and had undergone cryopreservation before CTC isolation. Cohort 2 included the 10 treatment naive patients from cohort 1. Patient characteristics of cohort 2 are summarised in Table 1. Twenty-four patients had resectable disease (Stage Ⅱ or Ⅲ). Post CTC evaluation, 11 of these patients received neoadjuvant chemoradiotherapy prior to resection (CROSS regimen), 3 received perioperative chemotherapy (MAGIC regimen), and 10 had surgery alone. Nineteen patients had metastatic disease (stage Ⅳ). Most of these patients received chemotherapy (7 patients: platinum and capecitabine doublet, 3 patients: anthracycline, capecitabine, and platinum triplet, 1 patient: irinotecan or paclitaxel monotherapy), immunotherapy (2 patients), and 6 patients received no active systemic treatments.

CTCs were detected in 42/43 patients (95.5%),

Table 1 Characteristics of patients in cohort 2 *n* (%)

	All patients	CTC count	
		Low (CTC ≤ 17)	High (CTC > 17)
	n = 43	*n* = 23	*n* = 20
Age			
Mean (range)	64 (39-89)	65 (39-89)	64 (48-83)
Sex			
Male	32 (74.4)	15 (65.2)	20 (85.0)
Female	11 (25.6)	8 (34.8)	3 (15.0)
ECOG			
0-1	36 (83.7)	22 (95.6)	14 (70.0)
2-4	7 (16.3)	1 (4.3)	6 (30.0)
Primary tumor location			
Distal oesophageal	12 (27.9)	8 (34.8)	4 (20.0)
Gastroesophageal junction	14 (32.6)	4 (17.4)	10 (50.0)
Gastric	17 (37.5)	11 (47.8)	6 (30.0)
Stage			
Ⅱ	18 (41.9)	13 (56.5)	5 (25.0)
Ⅲ	6 (14.0)	4 (17.4)	2 (10.0)
Ⅳ	19 (44.2)	6 (26.1)	13 (65.0)

CTC: Circulating tumor cell; ECOG: Eastern cooperative oncology group performance status.

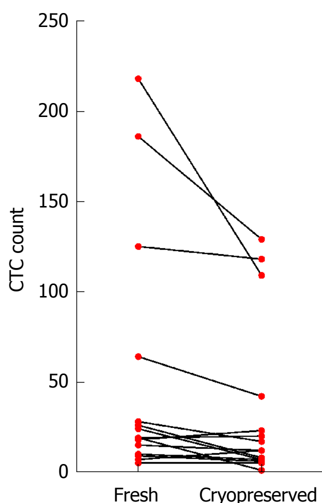

Figure 2 Circulating tumor cell enumeration by processing method. Mean number of CTCs isolated in the fresh specimens were higher than in the matched cryopreserved sample (mean difference in CTCs 17.1 95%CI: 0.7-33.6, *P* = 0.043). This difference was mostly driven by larger falls in CTC counts in samples with high numbers of CTCs (> 50 CTCs in fresh samples), with no significant difference in CTC counts for samples with less than 50 CTC in the fresh specimen (*P* = 0.06).

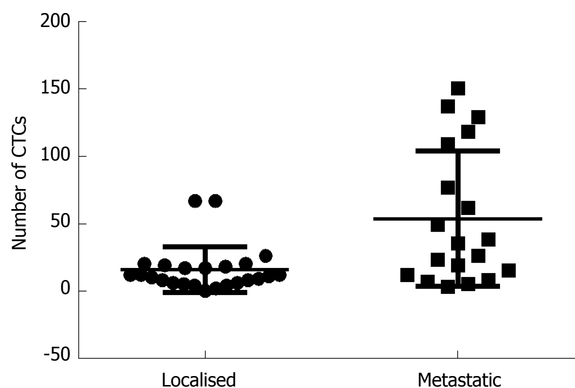

Figure 3 Circulating tumor cell count by stage. CTC processing post cryopreservation produced a higher mean CTC count in metastatic patients compared to the resectable patients (mean CTC in metastatic 53.8 *vs* resectable 15.8, *P* = 0.0013). CTC: Circulating tumor cell.

with a median CTC of 17 (interquartile range 8-38). Patients with metastatic disease had a higher number of CTCs than those with resectable disease (Figure 3, mean CTC count 53.8 *vs* 15.8, *P* = 0.0013).

Currently there are no established cut-offs for prognostic CTC numbers detected using the IsoFlux in gastroesophageal adenocarcinoma. Therefore we opted to divide our patients by their CTC counts, above versus equal or lower than the median CTC count, to test for any correlation with clinical outcomes. Patients with a high CTC count (> 17) had a poorer OS than those with a lower CTC count (≤ 17) (Figure 4, median OS 2.8 mo *vs* 23.2 mo, HR = 4.4, 95%CI: 1.7-11.7, *P* = 0.0013). In multivariate analysis, after controlling for sex, age, stage, ECOG performance status, and primary tumor location, a high CTC count remained an independent prognostic factor associated with poor OS (Table 2, HR = 3.7, 95%CI: 1.2-12.4, *P* = 0.03). This association was stronger when the analysis was restricted to patients with metastatic disease (*n* = 19, HR = 5.5, 95%CI: 1.2-25.5, *P* = 0.01), but not observed in patients with resectable disease (*n* = 24, *P* = 0.39), although a high CTC count (> 17) was associated with a non-significant trend to shorter recurrence free survival in these patients (HR = 3.1, 95%CI: 0.8-12.6, *P* = 0.09).

Most patients had some uPAR positive CTCs (40/43, 93.0%), however the proportion of uPAR positive CTCs was similar between patients with localised and metastatic disease (mean proportion uPAR positive CTCs 48.8% *vs* 47.7% respectively, *P* = 0.89), and there was no association with survival outcomes (Supplementary

Table 2 Univariate and multivariate analysis for overall survival for cohort 2 (*n* = 43)

Factor	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
CTC count (high *vs* low)	4.4 (1.7-11.7)	0.001	3.7 (1.2-12.4)	0.03
Age (≥ 65 *vs* <65 yr old)	0.7 (0.3-1.8)	0.46	1.0 (0.9-1.1)	0.76
ECOG (2-4 *vs* 0-1)	7.2 (2.2-23.7)	0.0002	2.3 (0.5-10.1)	0.14
Sex (male *vs* female)	1.2 (0.4-3.8)	0.7	0.7 (0.2-2.1)	0.49
Stage (Ⅳ *vs* Ⅱ-Ⅲ)	10.0 (3.3-30.8)	< 0.0001	9.9 (2.9-33.8)	0.0003
Primary tumor location (gastric *vs* oesophageal/GOJ)	0.3 (0.1-1.01)	0.05	0.4 (0.2-1.6)	0.22

Significant values are italicised. In both univariate and multivariate analysis, a high CTC count (> 17) remained statistically significant as an independent factor associated with poorer overall survival. CTC: Circulating tumor cell; ECOG: Eastern cooperative oncology group performance status; GOJ: Gastroesophageal junction.

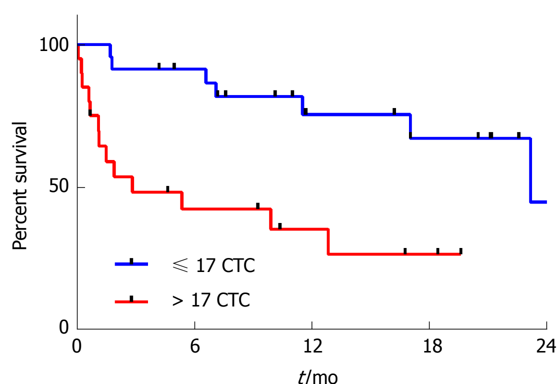

Figure 4 Overall survival by circulating tumor cell count. Patients with > 17 CTCs isolated from cryopreserved specimens had a poorer overall survival compared to those with ≤ 17 CTCs (median OS 2.8 mo *vs* 23.2 mo, HR = 4.4, 95%CI: 1.7-11.7, *P* = 0.0013). OS: Overall survival; CTC: Circulating tumor cell.

Figure 1, median OS 17.0 mo *vs* 12.8 mo, *P* = 0.6).

DISCUSSION

In this study we report the reliable isolation, immunocytochemical identification, and enumeration of gastroesophageal cancer CTCs from cryopreserved PBMCs using the IsoFlux platform. The included cohort is the largest reported study analysing cryopreservation of patient PBMCs for CTC detection. Our data confirms that CTCs isolated from cryopreserved samples remain an independent prognostic factor associated with OS.

The timely processing of patient samples for CTC isolation, usually is recommended within 24 h for most isolation methods[19], presenting significant logistical challenges for researchers and prohibits inclusion of patients from remote areas into clinical trials that would rely on CTCs as outcome measures. This is mainly because current methods of CTC analysis require significant expertise, instrumentation, time and laboratory resources, usually performed in specialised research centres. Protocols using isolation of CTCs from cryopreserved specimens would require some basic processing and cryopreservation at the site of blood draw, but offer many advantages, including the ability to biobank patient samples for prolonged periods of time before central processing. This would be a huge benefit for larger scale clinical trials as it would allow inclusion of geographically separated sites.

Previous work has shown that the immunochemical properties of CK, EpCAM and CD45, central to the isolation and identification of CTCs, are not affected by cryopreservation and thawing[20,21]. In agreement, our results demonstrate a similar morphological and immunofluorescent profile between cryopreserved and fresh CTCs and leukocytes, suggesting current techniques are suitable for cryopreserved samples. This approach is further supported by other work showing close concordance in genetic alterations seen on paired fresh and frozen CTCs[21].

Our results also show that enumeration of CTCs isolated from cryopreserved PMBCs is a valid prognostic biomarker in gastroesophageal cancer. Patients with metastatic disease had a significantly higher number of CTCs than those with resectable disease (mean CTC count 53.8 *vs* 15.8, *P* = 0.0013). Moreover, patients with a high CTC count (> 17) had a much poorer OS than those with a lower CTC count (≤ 17) (HR = 4.4, *P* = 0.0013). High CTC count remained significant in the multivariate analysis as an independent predictor of poorer OS (HR = 3.7, *P* = 0.03), after controlling for age, ECOG, sex, stage and primary tumour location, particularly when analysis was restricted to patients with metastatic disease only (HR = 5.5, *P* = 0.01). These results are concordant with other studies which confirm CTC enumeration as an important prognostic factor in gastroesophageal cancer[10-12].

Given our previous findings that the uPA system is a clinically relevant biomarker in primary gastroesophageal cancer[18], we undertook and successfully probed for uPAR expression in CTCs derived from cryopreserved and fresh samples. We previously have shown that higher expression of uPA, uPAR and PAI-1 in the primary tumour is associated with higher risk disease and poorer prognosis. However, in this study, there was no correlation between CTC uPAR expression with disease parameters. This suggests that the selection of epithelial (EpCAM-positive) CTCs might have affected any correlation of uPAR with patient outcome, as CTCs that present mesenchymal phenotypes, such as

uPAR expressing cells, can escape standard methods of isolation reliant on epithelial markers[22]. Indeed Vishnoi *et al*[23]. has previously reported the isolation of subsets of EpCAM-negative, uPAR and integrin β1 positive breast cancer CTCs, which further supports the concept of CTC heterogeneity[23]. Ultimately, we have successfully stained for a novel biomarker, uPAR, which further supports our cryopreservation method as a valid CTC isolation approach.

One important concern with cryopreservation is the potential for loss of CTCs due to cell loss during freezing, storage, or thawing. In a study by Nejlund *et al*[20], tumor cell recovery from cryopreserved spiked tumor cells in normal controls was variable, with up to a 40% tumor cell loss. However in clinical samples using matched fresh and cryopreserved specimens from the same patient, there was no consistent loss of CTCs, with the variation in CTC enumeration similar to those seen in paired fresh samples in other studies[2,20]. Friedlander *et al*[21] found that cryopreservation of PBMCs had no significant effect on the cell recovery from patients with metastatic prostate cancer. We noted a small loss of CTCs associated with cryopreservation, however this was predominately in samples with large numbers of CTCs (> 50), where loss of some CTCs is more acceptable than samples with low CTC counts. We noted samples with high numbers of CTCs were more prone to cell clumping despite benzonase. This is normally due to the release of viscous DNA from cell lysis on thawing, leading to aggregates which prevent accurate CTC counting. We speculate that the higher disease burden in these patients, coupled with a corresponding systemic inflammatory response, lead to poorer cell integrity within the PBMCs of high CTC-count samples. Some loss of CTCs in these samples will have little impact for prognostic and down-stream biomarker analysis purposes. There was no significant loss of CTCs in samples were the total CTC count was ≤ 50 (P = 0.06).

Similar to previously published work, we found that the duration of cryopreservation was not correlated with number of isolated CTCs[20]. Moreover, we were able to isolate CTCs from specimens stored at -80 ℃ for over two years, suggesting cryopreservation is a suitable approach for long term projects that involve biobanking of patient samples.

Even when using cryopreservation prior to CTC isolation, we found higher numbers of CTCs (median CTC count 17) and a higher number of patient samples with CTCs (98%) compared to other studies using EpCAM based CTC capture in gastroesophageal cancer[10-12,24]. The correlation of CTC numbers with disease progression implies that the CTCs we identified are indeed disease related. Increased CTC counts are consistent with the higher reported sensitivity of the IsoFlux system compared to other platforms, particularly in isolating CTCs with a lower expression of EpCAM[14-16]. Our results confirm, in the largest cohort of patients reported to date, that a high CTC count (> 17) in cryopreserved specimen was an independent prognostic factor associated with poorer OS (HR = 3.7). As expected from the minimal CTC loss during cryopreservation, these data indicate that indeed our method is suitable for delayed and centralised CTC analysis which could help recruiting patients for major clinical trials. In this setting it would be advantageous compared to fixation of blood which allows CTC processing delayed by only several days rather than long term biobanking. We are currently testing if cryopreservation is also able to overcome limitations associated with using fixative for molecular downstream analysis of CTCs that involves nucleic acid extraction[4,5].

In conclusion, we have tested a robust PBMC cryopreservation protocol that allows successful CTC isolation even 2 years post freezing. Cryopreservation of CTCs is feasible, with a small loss of tumor cells predominantly in samples with a high CTC load. Enumeration of CTCs from cryopreserved samples remained a clinically important prognostic biomarker. Cryopreservation may assist with the wider incorporation of CTC collection and analysis in biobanking, retrospective studies, and large international clinical trials, by facilitating specimen storage, bulk transporting, and batch processing. It may also help to develop diagnostic settings that can service even remote patients with diagnostic CTC data potentially relevant for their disease management.

ARTICLE HIGHLIGHTS

Research background

A persisting challenge to the field of circulating tumor cell (CTC) research is the requirement for prompt analysis of samples at specialised centres. This has presented significant logistical challenges to researchers, compounded by the significant expertise, time and laboratory resources required for CTC analysis.

Research motivation

Current methods to overcome this issue, such as fixation of blood samples, extend the time for CTC processing for several days, but may interfere with downstream molecular analyses.

Cryopreservation of patient samples permits the wider incorporation of CTC collection and analysis in biobanking, retrospective studies, and large international clinical trials, by facilitating specimen storage, bulk transporting, and batch processing. However, up to now, there has been little research in how cryopreservation affects CTC recovery, and whether cryopreservation retains predictive value of CTCs.

Research objectives

The primary objective of our study was to investigate the feasibility and reliability of delayed CTC isolation from cryopreserved peripheral blood mononuclear cells (PBMCs) layer. This was determined by percentage of CTC loss during cryopreservation and thawing, and clinical validity of CTC enumeration from cryopreserved samples.

Research methods

CTCs were isolated from 7.5 mL blood samples collected from patients with gastroesophageal adenocarcinoma using EpCAM based immunomagnetic capture with the IsoFlux platform. CTC loss with cryopreservation was determined by comparing CTC enumeration from matched cryopreserved

and freshly processed blood samples collected during the same blood draw. CTCs isolated from pre-treatment cryopreserved PBMCs were examined for association with clinicopathological variables and survival outcomes.

Research results

We found a minor loss of tumor cells in matched cryopreserved and freshly processed samples, mostly in samples with high CTC counts. A high CTC count isolated from cryopreserved PBMCs remained a statistically significant independent prognostic factor in gastroesophageal cancer.

Research conclusions

Our study demonstrates a feasible and robust protocol facilitating CTC isolation from cryopreserved PBMCs even after 2 years post freezing. Our results have immediate applicability in the design and conduct of translational studies, as it facilitates incorporation of CTC analysis in large international trials and biobanking projects.

Research perspectives

There is an increasing variety of techniques used for CTC isolation described in the literature. While the current work confirms the reliability of CTC isolation from cryopreserved samples using immunomagnetic separation, further work needs to be undertaken to confirm its suitability for other isolation approaches.

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RESEARCH ARTICLE Open Access

Sidedness is prognostic in locoregional colon cancer: an analysis of 9509 Australian patients

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Abstract

Background/Aim: Right sided colon cancer (RsCC) is proposed to be a distinct disease entity to left sided colon cancer (LsCC). We seek to confirm primary tumour location as an independent prognostic factor in locoregional colorectal cancer.

Methods: All patients with stage I – III primary adenocarcinoma of colon were identified from the New South Wales (NSW) clinical cancer registry (2006–2013). Primary tumour location (RsCC vs LsCC) survival analyses were conducted using the Kaplan-Meier method, and adjusted hazard ratios for 5-year all-cause mortality (OS) and 5-year cancer specific mortality (CSS) were obtained using Cox proportional hazards regression.

Results: We identified 9509 patients including 5051 patients with RsCC and 4458 with LsCC. Patients with RsCC were more likely to be older, female, have a higher Charlson comorbidity index, and have worse tumour prognostic factors. In univariate analysis of all stages combined, those patients with RsCC had a worse overall survival (OS, HR 1.20 95% CI 1.11–1.29, $p < 0.0001$), although this was not significant in the multivariate analysis (HR 0.96 95% CI 0.89–1.04, $p = 0.35$). Stage I patients with RsCC had a trend to improved OS (multivariate HR 0.84 95% CI 0.69–1.01, $p = 0.07$) and a significantly improved CSS (multivariate HR 0.51 95% CI 0.35–0.75, $p = 0.0006$). In stage II patients with RsCC there was a significantly improved OS (multivariate HR 0.85 95% CI 0.75–0.98, $p = 0.02$) and CSS (multivariate HR 0.59 95% CI 0.45–0.78, $p = 0.0002$) compared to LsCC. In stage III patients, those with RsCC had a worse OS (multivariate HR 1.13 95% CI 1.01–1.26, $p = 0.032$) and a trend to worse CSS (multivariate HR 1.12 95% CI 0.94–1.33, $p = 0.22$).

Conclusions: Primary tumour location is an important prognostic factor in locoregional colon cancer with an effect that varies by stage. RsCC is associated with lower all-cause mortality in stage II, and higher all-cause mortality in stage III.

Keywords: Colonic neoplasms/mortality, Colonic neoplasms/pathology, Neoplasm staging

Background

Colorectal (CRC) is a common and lethal malignancy, projected to account for 13% of all new cancer cases diagnosed in Australia in 2015, and 10% of Australian cancer deaths [1]. In recent years there has been increasing interest in identifying the differences between right sided and left sided colon cancer, and the potential for using this clinical marker as a surrogate marker of tumour biology, with the intent of improved personalisation of systemic treatments.

There is a growing body of evidence to suggest that right sided colon cancers (RsCC) follow a different disease process compared to left sided tumours (LsCC). The proximal and distal colons are physiologically separate, arising from distinct embryological origins, with differences in tumour genetics, histology, presentation, and clinical features [2–4]. Patients with RsCC are older, more likely to be female, have more comorbidities, with poorer tumour histopathological features [5–8].

Despite this, there is ongoing debate whether primary tumour location is an independent prognostic factor in

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colon cancer. Most, but not all studies have found poorer survival with RsCC [7–11]. A recent meta-analysis found a statistically significant worse overall survival in patients with RsCC, although there was significant heterogeneity seen due the spectrum of included study designs, disease stage, and limited information about treatment received by patients [12]. Tumour stage may play a role, with a large Surveillance, Epidemiology, and End Results (SEER) program study showing worse overall survival in Stage III RsCC patients, but not in Stage I or II [7], although these finding have been recently challenged by a propensity score matched analysis of the SEER database, which showed a better prognosis in RsCC patients [9].

This current study aims to use a prospectively collected database of Australian patients to determine whether primary tumour location is an independent prognostic factor in locoregional colon cancer, and compare our findings to the literature.

Methods

Patient cohort

The New South Wales (NSW) clinical cancer registry contains demographic and clinical data for patients diagnosed or treated for cancer in NSW, covering approximately 30% of the Australian population. Data is collected from pathological laboratories, hospitals and oncology departments under mandatory notification of new cancer cases irrespective of treatment.

We identified all patients with Stage I, II or III colorectal cancer in NSW from Jan 2006 to 2013 (n = 9509) as per third edition of the International Classification of Diseases for Oncology (ICD-O-3) [13]. The registry also contained adjuvant chemotherapy treatment details for a more limited group of patients with stage II and III disease (n = 4102).

Mortality data, including cause of death, was obtained with linkage to the NSW registry of Births, Deaths and Marriages (BDM) by the Centre for Health Record Linkage (CHeReL) [14]. The censor data for survival data was 1st December 2014. Primary tumour location was defined right sided (caecum to transverse colon) or left sided (splenic flexure to rectosigmoid). Patients with rectal cancer were excluded from analysis due to the different treatment paradigm to colon cancer in locoregional disease. No data was available for cause of death in 935 patients (10.1%) which were therefore excluded from the cancer specific death analyses. Patients were deemed to have died as a result of colon cancer only if the underlying cause of death, rather than an associated cause of death, was coded as C18–20.

Comorbidity data was obtained by CHeReL linkage of the clinical cancer registry data to the Admitted Patient Data Collection (APDC). The APDC contains all admitted patient services provided by New South Wales Public Hospitals, Public Psychiatric Hospitals, Public Multi-Purpose Services, Private Hospitals, and Private Day Procedures Centres. Comorbidities of each patient were quantified using the Charlson comorbidity index which predicts mortality from a range of 22 comorbid conditions [16]. ICD-10 codes were extracted from admissions prior to diagnosis, then translated into a Charlson comorbidity index (modified for cancer) using methods previously described [15, 16].

All data linkage was performed by the Centre for Health Record Linkage, with only de-identified information provided to the researchers. The data sources used for this study required ethical and data custodian approval to access, link (by an independent and approved authority) and release for research. Approval for this project was provided by the NSW Population & Health Services Research Ethics Committee (approval HREC/13/CIPHS/39).

Statistical analysis

Our primary outcome was all-cause 5-year overall survival (OS) stratified by stage, defined as death within 5 years of primary diagnosis of colon cancer on basis of dates recorded in the cancer registry and BDM databases. The secondary outcome was cancer specific 5 year survival (CSS) stratified by stage, as per cause of death encoded on BDM data. Median values for OS and CSS-OS and corresponding 95% CI were calculated using Kaplan-Meier methods. Unadjusted and multivariable Cox proportional hazards regression analyses were used to estimate the association between tumour location and survival and to calculate corresponding hazard ratios (HRs) and 95% confidence intervals (CIs). The following variables were included in the multivariate model: age, sex, Charlson Comorbidity Index, TNM stage, year of diagnosis, grade, and adjuvant treatment (receipt and type of adjuvant treatment performed in subset of patients only). All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc., Cary, NC).

Results

Patient characteristics (n = 9509)

The characteristics of the NSW cohort is summarised in Table 1. The mean follow up was 46 months (interquartile range 27 to 71 months). At the end of 5 years of follow up, 2686 (28.2%) patients had died, with 913 reported deaths (34.0% of deaths) due to colon cancer. 22% of patients had stage I disease, 39% stage II, and 39% had Stage III. There were slightly more RsCC (53%) than LsCC (47%). Patients with RsCC were older (61% vs 47% older than 70 years), more likely to be female (54% vs 42% female), had higher Charlson comorbidity indices (CCI, 40% vs 34% CCI ≥ 1), and had worse

Table 1 Patient characteristics (n = 9509)

Characteristic		All Patients (%)	Right sided tumour (%)	Left sided tumour (%)	*P* value
TNM stage	I	2104 (22)	1055 (21)	1049 (24)	<0.0001
	II	3684 (39)	2059 (41)	1625 (36)	
	III	3721 (39)	1937 (38)	1784 (40)	
T stage	1	1526 (16)	715 (14)	811 (18)	<0.0001
	2	1030 (11)	558 (11)	472 (11)	
	3	5075 (53)	2741 (54)	2334 (52)	
	4	1868 (20)	1031 (20)	837 (19)	
N Stage	0	5788 (61)	3114 (62)	2674 (60)	0.06
	1	3065 (32)	1576 (31)	1489 (33)	
	2	656 (7)	361 (7)	295 (7)	
Grade	Well differentiated	1244 (13)	635 (13)	609 (14)	<0.0001
	Mod. differentiated	6648 (70)	3278 (65)	3370 (76)	
	Poorly Differentiated	1617 (17)	1138 (23)	479 (11)	
Age group	≤60	1925 (20)	798 (16)	1127 (25)	<0.0001
	61–70	2423 (25)	1189 (24)	1234 (28)	
	71–80	2814 (30)	1600 (32)	1214 (27)	
	>80	2347 (25)	1464 (29)	883 (20)	
Sex	Male	4913 (52)	2317 (46)	2596 (58)	<0.0001
	Female	4596 (48)	2734 (54)	1862 (42)	
Charlson Comorbidity Index	0	5957 (63)	3027 (60)	2930 (66)	<0.0001
	1–2	5083 (22)	1172 (23)	911 (20)	
	3–4	1023 (11)	596 (12)	427 (10)	
	5	446 (5)	256 (5)	190 (4)	
Adjuvant Chemotherapy	None	1775 (19)	955 (46)	820 (40)	0.0002
	Fluorouracil based	1098 (12)	553 (27)	545 (27)	
	Oxaliplatin doublet	1233 (13)	568 (27)	665 (33)	
	Unknown[a]	5403	2975	2428	
Year Diagnosed	2006–2009	5018 (53)	2644 (52)	2374 (53)	0.38
	2010–2013	4491 (47)	2407 (48)	2084 (47)	
Totals		9509	5051 (53)	4458 (47)	

[a]Not included in multivariate analysis in chemotherapy cohort

prognostic features including higher TNM stage (79% vs 76% stage II/III), and higher grade tumour (23% vs 11% poorly differentiated).

5 year all-cause mortality by primary tumour location

The observed 5 year OS for patients with RsCC was 66% (95% CI 65–67%) compared to 70% (95% CI 69–72%) for LsCC. Unadjusted survival analysis demonstrated a higher mortality with RsCC in all stages combined (Fig. 1, univariate HR 1.20 95% CI 1.11–1.29, p < 0.0001). When stratified by stage there was significant difference in OS seen only in stage III, with a higher mortality seen in RsCC (Fig. 1, HR 1.46 95% CI 1.31–1.63, p < 0.0001) (Fig. 1).

After adjusting for sex, age, comorbidities, stage, grade, and year of diagnosis there was no significant difference in OS between RsCC and LsCC in patients from all stages (multivariate HR 0.96 95% CI 0.89–1.04 p = 0.35) (Table 2). When the multivariate analysis was stratified by stage, patients with RsCC had a trend to improved survival in stage I (HR 0.84 95% CI 0.69–1.01, p = 0.069), a statistically significant improved survival in stage II (HR 0.85 95% CI 0.75–0.98, p = 0.02), but a shorter survival in stage III (HR 1.13 95% CI 1.01–1.26, p = 0.03) (see Table 3.)

Cancer specific survival (CSS) primary tumour location

The 5 year cancer specific survival (CSS) was similar for RsCC (89%; 95% CI 88–90%) and LsCC (89%; 95% CI

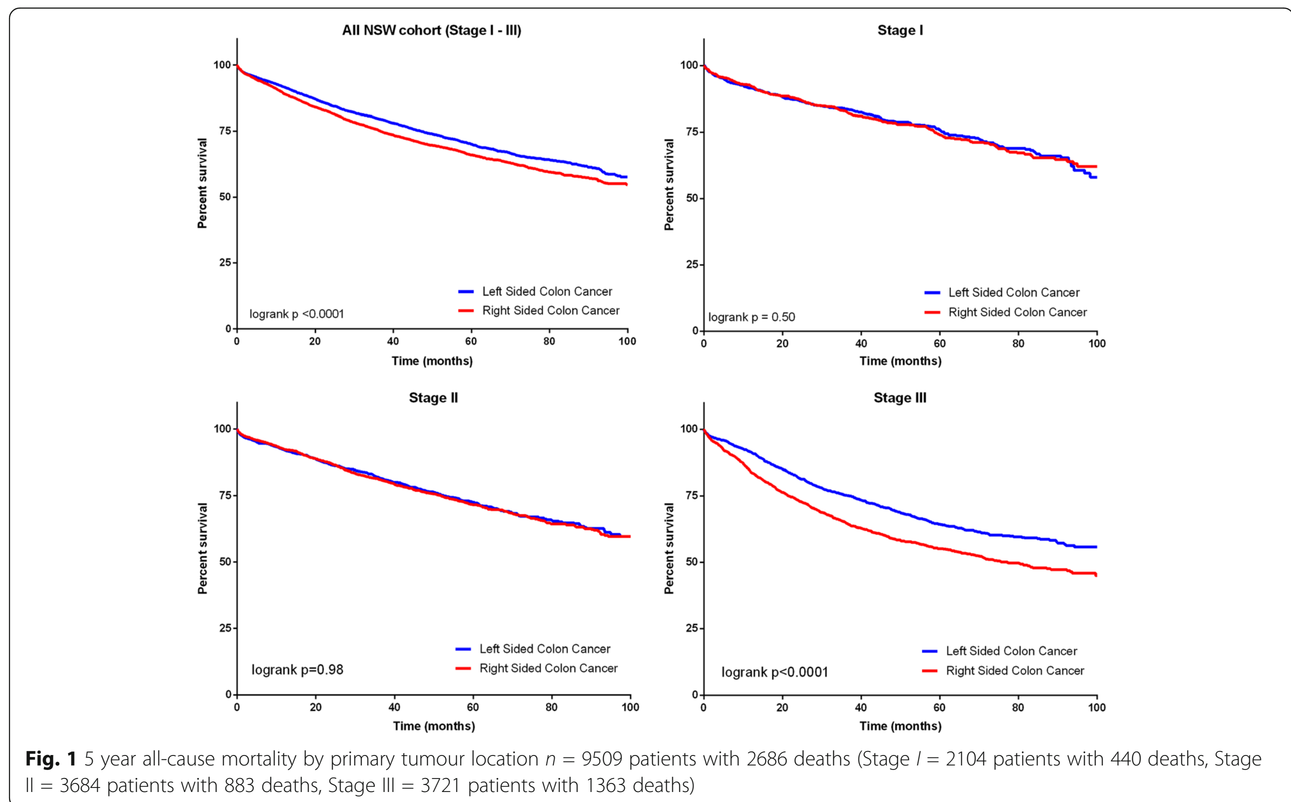

Fig. 1 5 year all-cause mortality by primary tumour location n = 9509 patients with 2686 deaths (Stage *I* = 2104 patients with 440 deaths, Stage II = 3684 patients with 883 deaths, Stage III = 3721 patients with 1363 deaths)

Table 2 Multivariate model for overall survival for NSW cohort (n = 9509)

Characteristic		Multivariate HR (95% CI)
Sided	Left	1
	Right	0.96 (0.89–1.04)
Age	≤60	1
	61–70	1.34 (1.15–1.56)
	71–80	2.23 (1.93–2.56)
	>80	3.97 (3.46–4.56)
Grade	Well differentiated	1
	Moderately differentiated	1.22 (1.06–1.39)
	Poorly Differentiated	1.87 (1.60–2.17)
TNM stage	I	1
	II	1.05 (0.96–1.21)
	III	2.00 (1.80–2.24)
Sex	Male	1
	Female	0.90 (0.83–0.97)
Charlson Comorbidity Index	0	1
	1–2	1.64 (1.49–1.79)
	3–4	1.81 (1.62–2.03)
	5	3.02 (2.63–3.46)
Year Diagnosed	2006–2009	1
	2010–2013	0.98 (0.90–1.06)

HR Hazard Ratio, *CI* confidence interval

87–90%). Unadjusted CSS analysis did not show a significant difference between RsCC and LsCC in all stages combined (Fig. 2, univariate HR 1.03 95% CI 0.91–1.18, p = 0.64). When stratified by stage, there was a significantly improved CSS seen with RsCC in stage I (HR 0.66 95% CI 0.45–0.95, p = 0.024) and stage II (HR 0.68 95% CI 0.52–0.88 p = 0.0032), but a significantly poorer survival for stage III patients (HR 1.43 95% CI 1.21–1.66, p < 0.0001) (Fig. 2, Table 3).

In the multivariate analysis, after adjusting for sex, age, comorbidities, stage, grade, and year of diagnosis, patients with RsCC had a statistically significant improved CSS in all stages combined (HR 0.84, 95% CI 0.73–0.96, p = 0.011), and for stage I (HR 0.51 95% CI 0.35–0.75, p = 0.0006) and stage II (HR 0.59 95% CI 0.45–0.78, p = 0.0002) patients, but a trend to worse survival in stage III (HR 1.12 95% CI 0.94–1.33, p = 0.22) (Table 3).

Effect of adjuvant chemotherapy

Adjuvant treatment details were available for 1631 (44%) of patients with stage II and 2441 (66%) of patients with stage III disease (4102 patients total). Most patients in stage II disease did not receive adjuvant chemotherapy (72%), with only a minority receiving fluorouracil monotherapy (24%) or an oxaliplatin doublet combination (usually FOLFOX, 5%). In contrast, the majority of patients with stage III disease received adjuvant chemotherapy

Table 3 Univariate and multivariate Hazard Ratios for NSW cohort (n = 9509) stratified by stage. Statistically significant values in bold

		Overall Survival HR (95% CI)		Cancer Specific Survival HR (95% CI)	
		Univariate	Multivariate[a]	Univariate	Multivariate[a]
All Patients	Left Sided	**1**	1	1	**1**
	Right Sided	**1.20 (1.11–1.29)**	0.96 (0.89–1.04)	1.03 (0.91–1.18)	**0.84 (0.73–0.96)**
Stage I (n = 2104)	Left Sided	1	1	**1**	**1**
	Right Sided	1.03 (0.91–1.18)	0.84 (0.69–1.01)	**0.66 (0.45–0.95)**	**0.51 (0.35–0.75)**
Stage II (n = 3684)	Left Sided	1	**1**	**1**	**1**
	Right Sided	1.002 (0.88–1.14)	**0.85 (0.75–0.98)**	**0.68 (0.52–0.88)**	**0.59 (0.45–0.78)**
Stage III (n = 3721)	Left Sided	**1**	**1**	**1**	1
	Right Sided	**1.46 (1.31–1.63)**	**1.13 (1.01–1.26)**	**1.43 (1.21–1.69)**	1.12 (0.94–1.33)

[a]Following variables were used in the multivariate analysis: age, sex, year diagnosed, Charlson Comorbidity Index, TNM stage, grade

(75%), with 28% treated with fluorouracil monotherapy, and 47% with an oxaliplatin/ fluorouracil doublet. Higher TNM-substage was associated with treatment with oxaliplatin doublet within both stage II (p < 0.0001) and III (p = 0.0001). Consistent with current practice no patients received adjuvant treatment with monoclonal antibodies.

Patients with RsCC were less likely to receive adjuvant chemotherapy (p = 0.0002, Table 1) despite higher risk tumour features. Adjuvant chemotherapy improved survival in both RsCC (univariate OS HR 0.68; 95% CI 0.58–0.80) and LsCC (univariate OS HR 0.48; 95% CI 0.40–0.58, Additional file 1: Figure S1 and Additional file 2: Figure S2.

Inclusion of the adjuvant chemotherapy regimen into the multivariate model did not alter the effect of primary tumour location, although the results for RsCC in stage II disease became non-significant (multivariate OS HR 0.86 95% CI 0.69–1.09 p = 0.19; multivariate CSS HR 0.67 95% CI 0.43–1.04, p = 0.07, Table 4). Patients with RsCC in stage III colon cancer continued to have a significantly inferior OS compared to LsCC even after adjustment for all above factors including receipt and type of adjuvant chemotherapy (multivariate OS HR 1.29 95% CI 1.11–1.50 p = 0.0012; multivariate CSS HR 1.16 95% CI 0.92–1.47, p = 0.22, Table 4). When analyses were

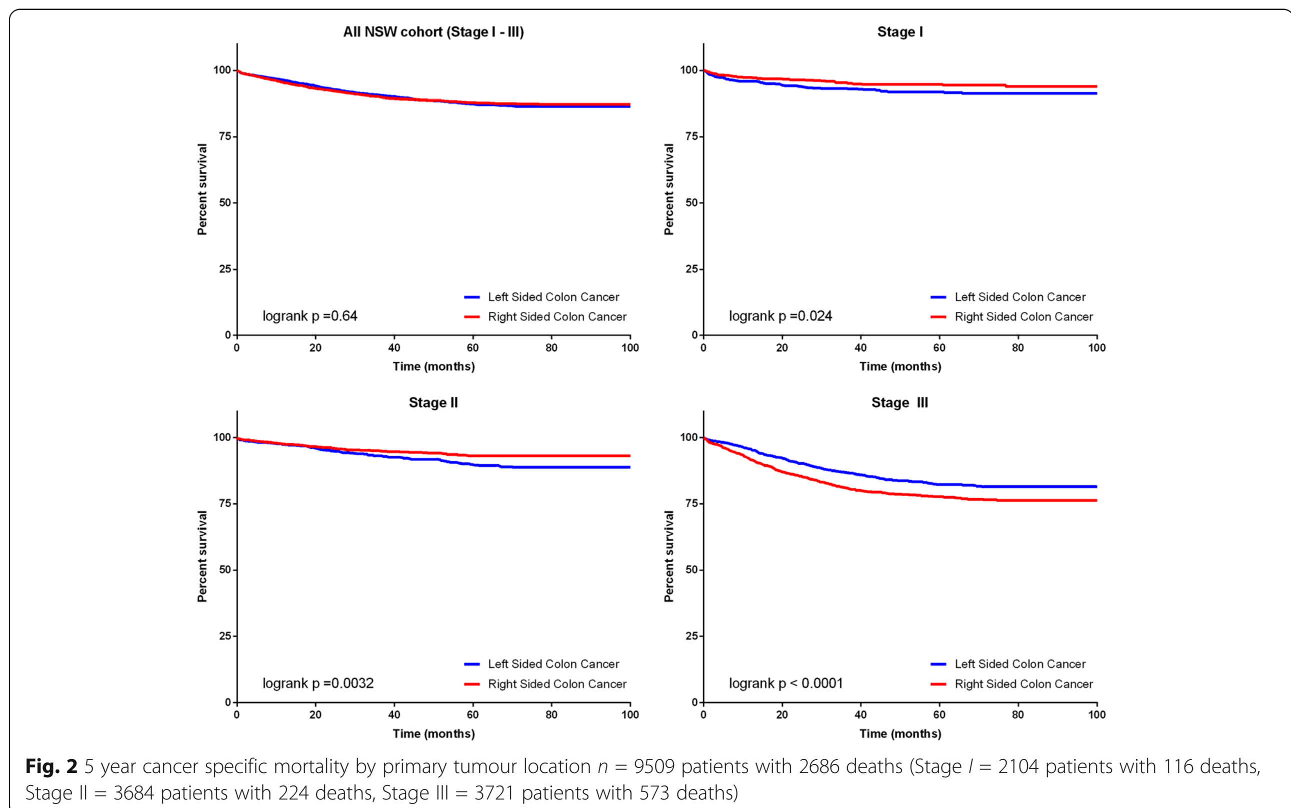

Fig. 2 5 year cancer specific mortality by primary tumour location n = 9509 patients with 2686 deaths (Stage I = 2104 patients with 116 deaths, Stage II = 3684 patients with 224 deaths, Stage III = 3721 patients with 573 deaths)

Table 4 Multivariate model for overall survival for chemotherapy cohort (n = 4102)

Characteristic		Stage II (n = 1631)	Stage III (n = 2441)
		Multivariate	Multivariate
		HR (95% CI)	HR (95% CI)
Sided	Left	1	1
	Right	0.86 (0.68–1.09)	1.29 (1.11–1.50)
Age	≤60	1	1
	61–70	1.90 (1.20–2.99)	1.21 (0.94–1.54)
	71–80	2.97 (1.92–4.58)	1.81 (1.43–2.30)
	>80	5.92 (3.82–9.19)	2.00 (1.54–2.60)
Grade	Well/mod differentiated	1	1
	Poorly Differentiated	1.43 (1.08–1.90)	1.49 (1.26–1.75)
TNM stage	IIIa	1	1
	IIIb	2.20 (1.71–2.82)[a]	1.79 (1.33–2.43)
	IIIc	-	3.86 (2.84–5.24)
Sex	Male	1	1
	Female	0.85 (0.68–1.07)	0.94 (0.82–1.10)
CCI	0	1	1
	1–2	1.42 (1.09–1.52)	1.15 (0.96–1.38)
	3–4	1.60 (1.12–2.28)	1.20 (0.94–1.53)
	5	2.31 (1.45–3.69)	1.83 (1.36–2.46)
Year Diagnosed	2006–2009	1	1
	2010–2013	0.99 (0.79–1.26)	1.00 (0.86–1.17)
Adjuvant Chemotherapy	Nil	1	1
	Fluorouracil monotherapy	0.79 (0.51–1.10)[b]	0.48 (0.40–0.57)
	Oxaliplatin doublet	-	0.38 (0.27–0.42)

HR Hazard Ratio, *CI* confidence interval, *CCI* Charlson Comorbity index
[a]IIa vs IIb/IIc, [b]chemotherapy vs no chemotherapy

restricted to only those stage III patients who received adjuvant oxaliplatin doublet chemotherapy (n = 1233), RsCC remained associated with a poorer OS (univariate OS HR 1.8 95% CI 1.4–2.4, $p < 0.0001$).

Discussion

There are well established differences in patient demographics, tumour factors and clinical presentation between RsCC and LsCC [7, 9, 10, 17, 18]. However it remains uncertain whether primary tumour location is an independent prognostic factor in locoregional colon cancer.

The strongest evidence comes from a recent meta-analysis of 66 studies including 1,437,846 patients which showed LsCC is associated with a significantly reduced risk of death compared to RsCC (HR 0.82; 95% CI 0.79–0.84, $P < 0.01$) [12]. This study included all stages of colon cancer and found that, based on meta-regression, the effect of primary tumour location was independent of stage, race, year of study, and quality of study.

It is important to consider the limitations of the above meta-analysis. Firstly, there was significant heterogeneity seen in the results (I^2 = 93%), which is likely due to the variety of included study designs, differing multivariate covariates from source studies, and patient populations, with the estimate derived from overall populations with no stratification by stage.

Secondly, while most of the included studies controlled for tumour factors (such as stage and grade), and patient demographic factors (eg., age, sex), only three studies included a comorbidity index in the multivariate model [7, 17, 19], and only 21% (14 of 66 studies) included performance status. RsCC is more likely to occur in older patients who have more associated comorbidities [17], and the substantial imbalances in the baseline characteristics between LsCC and RsCC patients in these trials may be an unmeasured confounder which explains the improved survival with LsCC. This issue has been directly addressed by Warschkow et al. [9] who, in order to minimise confounding, used propensity score matching to analyse survival in RsCC versus LsCC in 91,416 patients with stage I-III colon cancer from the SEER database. These authors showed that RsCC had a better OS (HR 0.89, $p < 0.001$) and CSS (HR 0.71, $p < 0.001$) in stage I and II, but a similar prognosis in stage III (OS HR 0.99, p = 0.49; CSS HR 1.04, p = 0.129).

Our current study, using a large series of Australian patients from a prospectively collected database, and controlling for patient factors (including comorbidities), tumour factors, and adjuvant chemotherapy, confirmed previous studies showing that RsCCs are more likely to have a more advanced stage ($p < 0.0001$) and grade ($p < 0.0001$), and occur in older patients ($p < 0.0001$) with more comorbidities ($p < 0.0001$). Despite higher risk tumour features, patients with RsCC are less likely to receive adjuvant chemotherapy ($p < 0.0001$) or oxaliplatin doublet chemotherapy (p = 0.0002).

In the survival analysis, patients with RsCC have a lower all-cause mortality in stage II (HR 0.85, p = 0.02), but a higher mortality in stage III (HR 1.13, p = 0.032). Moreover, patients with RsCC had an improved 5-year CSS in Stage I (HR 0.51,p = 0.0006) and Stage II (HR 0.59, p = 0.0002), and a trend to inferior CSS in Stage III.

As adjuvant chemotherapy has been shown to have a larger benefit in RsCC than LsCC [20], we subsequently undertook further multivariate analysis in a subset of patients with known adjuvant chemotherapy protocols to validate our findings. Adjuvant chemotherapy improved survival in both RsCC and LsCC. We found incorporation of adjuvant chemotherapy into the multivariate

model did not alter the effect of primary tumour location. Although definitive conclusions were limited in stage II as chemotherapy regimens where only available in 44% of patients, there were similar hazard ratios showing improved OS and CSS with RsCC (multivariate HR 0.86 and 0.67 respectively), although statistically non-significant in the chemotherapy cohort. In stage III, where chemotherapy data was available for the majority of patients (66%), the results of multivariate analysis was very similar to overall cohort, with a significantly higher all-cause mortality with RsCC (HR 1.29, $p = 0.0012$) and trend to higher cancer specific mortality (HR 1.16, $p = 0.21$).

Our findings are consistent with the results of Wiess et al. [7], a large multivariate retrospective analysis of 53,801 patients from the SEER database linked to Medicare data, and controlled for comorbidities using Hierarchical Condition Categories risk score. Similar to our findings, in multivariate analysis, patients with RsCC had a non-significant trend to lower mortality in stage I (HR 0.95, $p = 0.21$), a lower mortality in stage II (HR 0.92, $p < 0.0001$), but a higher mortality in stage III (HR 1.12, $p < 0.001$), and a non-significant difference in mortality overall (HR 1.01, $p = 0.60$). This stage dependant effect, with an improved survival in RsCC in stage II, but higher mortality in stage III, has been reported by multiple other series [8–10, 18, 21].

The cause of the demonstrated inconsistent effect of primary tumour location by stage is unclear. Our study, and the quoted literature, are retrospective analyses of large population databases, and are susceptible to the inherent bias of confounding associated with this study design. However an alternative explanation to consider is the increasingly described differences in tumour biology between RsCC and LsCC. RsCCs are more likely to have adverse histological features (such as advanced T stage, higher grade, or lymophvascular invasion) and mucinous histology [2, 22–24]. Perhaps more importantly, there are also marked differences in the molecular profile between these tumours [25]. RsCC has a higher rate of BRAF mutations and high microsatellite instability (MSI-H), both which have established prognostic importance, with MSI-H tumours shown to have a favourable prognosis, and BRAF a strong poor prognostic marker in non-MSI-H but not in MSI-H tumours [22, 23, 26, 27]. In addition even within MSI-H tumours there are known differences in prognosis, with hereditary MSI-H colon cancers shown to have a better survival than sporadic cases [28]. It is important to note that these biomarkers are not uniformly distributed by stage, with MSI-H tumours associated with lower stage (21% in stage II vs 14% stage III and 4% stage IV), and BRAF mutant tumours more likely to occur at a higher stage [22, 29, 30]. Furthermore, previous studies have shown a differential effect of adjuvant chemotherapy in between molecular subtypes. There is a reduced benefit with fluorouracil based chemotherapy in MSI-H tumours, but preserved efficacy of oxaliplatin in MSI-H stage III colon cancer patients [31, 32]. Although our study demonstrated a persistent effect of primary tumour location even when OS analysis was restricted to those patients who received adjuvant oxaliplatin doublet chemotherapy, it is important to note that fewer patients with RsCC received oxaliplatin as part of the adjuvant treatment.

Therefore, in the absence of both family history and molecular profiles in these population series, it is reasonable to hypothesise that some of the observed survival difference in stage II and III may be due to unequal distribution of these biomarkers. However, emerging evidence suggests that primary tumour location may be a clinical surrogate for further, yet unidentified, predictive biomarkers as highlighted by the recent data from the FIRE3 and CALGB/SWOG 80405 trials, which suggests a reduced benefit to anti-EGFR treatment in RsCC independent of currently identified biomarkers [33]. A limitation of our study is the lack of associated molecular data which is a potential source of unmeasured confounding to the results.

Conclusion

This population based study provides further evidence that primary tumour location is an important independent clinical prognostic factor in stage II and III colon cancer with immediate implications for clinical practice and trial design. This clinical biomarker is likely acting as a surrogate for as yet unidentified molecular factors. Further studies with associated tumour molecular profiles are required to clarify the underlying biological differences between RsCC and LsCC.

Additional files

Additional file 1: Figure S1. Effect of adjuvant chemotherapy on overall survival in patients with right sided colon cancer. Description: Overall survival in patients with right sided colon cancer by receipt of adjuvant chemotherapy ($n = 2076$). (TIFF 40 kb)

Additional file 2: Figure S2. Effect of adjuvant chemotherapy on overall survival in patients with left sided colon cancer. Description: Overall survival in patients with left sided colon cancer by receipt of adjuvant chemotherapy ($n = 2030$). (TIFF 40 kb)

Abbreviations

CSS: Cancer specific survival; HR 95% CI: Hazard ratios and 95% confidence intervals; LsCC: Left sided colon cancer; MSI-H: High microsatellite instability; NSW: New South Wales; OS: Overall survival; RsCC: Right sided colon cancer

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Availability of data and materials
The datasets generated and/or analysed during the current study are not publicly available as it contains de-identified patient data.

Authors' contributions
Conception and design: DB, MR, MA, PdS, PC. Acquisition of data: EH, JR, TT, MA, PdS. Analysis of data: DB. Interpretation of data: DB, MA, PdS, WN, WC, MC, PC, MR. All authors contributed to manscuript drafing or revising. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All data linkage was performed by the Centre for Health Record Linkage, with only de-identified information provided to the researchers. While patient consent was not required for this study as only de-identified data was provided, the data sources used for this study required ethical and data custodian approval to access, link (by an independent and approved authority) and release for research. Approval for this project was provided by the NSW Population & Health Services Research Ethics Committee (approval HREC/13/CIPHS/39). Site specific approval was given by the Area Health Services that entered data into clinical cancer registry: South East Sydney Illawarra Area Health Service; Sydney West Area Health Service; North Sydney Central Coast Area Health Service; Hunter New England Area Health Service; North Coast Area Health Service; South Western Sydney Area Health Service.

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Safety and Efficacy of Oxaliplatin Doublet Adjuvant Chemotherapy in Elderly Patients With Stage III Colon Cancer

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Abstract

Owing to poor representation in trials, the optimum adjuvant regimen for elderly patients with stage III colon cancer is uncertain. We employed data from a cancer registry to show a survival benefit with the addition of oxaliplatin to fluoropyrimidine in patients ≥ 70 years. We note an increased rate of hospital admissions and early chemotherapy cessation in elderly patients on oxaliplatin.

Background: Colon cancer is common in the elderly, but owing to under representation in clinical trials, the benefit of standard therapies is uncertain in this age group. We aimed to clarify the efficacy and complications of adjuvant oxaliplatin and fluoropyrimidine chemotherapy for patients 70 years and older with stage III colon cancer. **Patients and Methods:** All patients with stage III colon adenocarcinoma were identified from an Australian cancer registry (2006-2013). Multivariable Cox hazard regression was used to determine prognostic factors for all-cause mortality. Chemotherapy complications were quantified using discontinuation rates, hospital admissions, and mortality for 12 months after starting chemotherapy. **Results:** A total of 2164 patients fulfilled our inclusion criteria, including 1080 (49.9%) patients $\geq$ 70 years. Patients $\geq$ 70 years were less likely to receive adjuvant chemotherapy (60.7% vs. 89.6%) or oxaliplatin doublet chemotherapy (18.8% vs. 71.2%). Older patients receiving oxaliplatin were more likely to cease treatment early (18.7% vs. 7.6%) and require hospital admission (67.0% vs. 53.5%). The addition of oxaliplatin provided an overall survival benefit for patients < 70 years (hazard ratio, 0.44; 95% confidence interval, 0.3-0.6; $P < .0001$) and for patients $\geq$ 70 years (hazard ratio, 0.64; 95% confidence interval, 0.5-0.9; $P = .005$). **Conclusions:** Despite a modestly increased rate of hospital admission and early chemotherapy cessation, we demonstrate a persistent survival benefit for the addition of oxaliplatin to a fluoropyrimidine as adjuvant treatment for stage III colon cancer in elderly patients.

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Keywords: Aged, Age groups, Colonic neoplasms, Comorbidity, Medical record linkage

Introduction

Colon cancer is a common and lethal malignancy, with about 100,000 new cases diagnosed annually in the United States.[1] It is a disease related to aging, with almost 40% of colon cancer diagnosed in patients > 75 years.[2-4]

Surgical resection is the only curative treatment for locoregional disease, although many patients will develop disease recurrence owing to micrometastases present at surgery. In resected stage III colon cancer, standard treatment includes adjuvant doublet chemotherapy with oxaliplatin and a fluoropyrimidine,[5] following the results of several large phase III randomized controlled trials that showed a 30% reduction in disease recurrence and 22% reduction in risk of death with the addition of oxaliplatin to fluoropyrimidine alone.[6-8]

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Table 1 Patient Characteristics

	All Patients, n = 2164 (%)	Patients < 70 Years, n = 1084 (50.1%)	Patients ≥ 70 Years, n = 1080 (49.9%)	*P* Value
TNM stage				
IIIa	272 (12.6)	141 (13.0)	131 (12.1)	.81
IIIb	1284 (59.3)	638 (59.9)	646 (59.8)	
IIIc	608 (28.1)	305 (28.1)	303 (28.1)	
Charlson comordibity index				
0	1485 (68.6)	831 (76.7)	654 (60.6)	< .0001
1-2	403 (18.6)	165 (15.2)	238 (22.1)	
3-4	195 (9.0)	63 (5.8)	132 (12.2)	
5 or more	81 (3.7)	25 (2.3)	56 (5.2)	
Primary tumor location				
Right	1053 (48.7)	463 (42.7)	590 (54.6)	< .0001
Left	1111 (51.3)	621 (57.3)	490 (45.4)	
Age group, y				
< 60	513 (23.7)	513 (47.3)		
60-69	571 (26.4)	571 (52.7)		
70-79	709 (32.8)		709 (65.7)	
≥ 80	371 (17.1)		371 (34.3)	
Gender				
Male	1125 (52.0)	577 (53.2)	548 (50.8)	.25
Female	1039 (48.0)	507 (46.8)	532 (49.3)	
Grade				
Well-differentiated	220 (10.2)	112 (10.3)	108 (10.0)	.0028
Moderately differentiated	1484 (68.6)	774 (71.4)	710 (65.7)	
Poorly differentiated	460 (21.3)	198 (18.3)	262 (24.3)	
Year diagnosed				
2006-2009	1096 (50.7)	570 (52.6)	526 (48.7)	.07
2010-2013	1068 (49.4)	514 (47.4)	554 (51.3)	
Adjuvant chemotherapy				
None	538 (24.9)	113 (10.4)	425 (39.4)	< .0001
Fluoropyrimidine monotherapy	651 (30.1)	199 (18.4)	452 (41.8)	
Oxaliplatin doublet	975 (45.0)	772 (71.2)	203 (18.8)	

Elderly patients appear to gain a similar benefit to fluoropyrimidine-based adjuvant chemotherapy compared with younger patients.[9] However, as only a minority of patients in clinical trials are older than 70 years, the efficacy and safety of adjuvant chemotherapy with an oxaliplatin doublet in elderly patients is unclear. For example, in the Adjuvant Colon Cancer End Points (ACCENT) database, which includes individual patient data from 14,500 participants in 18 fluoropyrimidine-based adjuvant trials, only 18% are older than 70 years.[10]

Currently available trial data is conflicting. Subgroup analyses from the pivotal phase III MOASIC and NSABO-07 trials show a survival benefit only in patients < 70 years.[6,7] Similarly, there was no disease-free survival (DFS) or overall survival (OS) improvement with the addition of oxaliplatin in the 2575 patients ≥ 70 years in the ACCENT database.[10] In contrast, however, pooled individual patient data from 904 patients ≥ 70 years from the NSABP C-08, XELOXA, X-ACT, and AVANT studies showed an attenuated but statistically significant benefit to the addition of oxaliplatin, including those with comorbidities.[11]

Similarly, retrospective patient series demonstrate contrasting results. Although the largest series, drawn from multiple United States databases including the Surveillance, Epidemiology, and End Results (SEER) database, found a statistically significant benefit to adjuvant oxaliplatin in elderly patients (70-74 years old) and those with comorbidities,[12] this was less clear in patients > 75 years[13] and was not seen in other, smaller studies.[14,15]

As a consequence of these uncertainties, current guidelines recommend discussing the incorporation of oxaliplatin with patients over 70 years based on individual circumstances, although fluoropyrimidine monotherapy is an appropriate choice for adjuvant therapy in the elderly.[5,16]

The current study employs data from an Australian cancer registry to investigate the comparative effectiveness of the addition of oxaliplatin to fluoropyrimidine monotherapy as adjuvant treatment

Table 2 Univariate and Multivariate Analyses for Overall Survival in all Patients Who Received Adjuvant Chemotherapy (N = 1626)

Characteristic	Univariate HR (95% CI)	*P* Value	Multivariate (95% CI)	*P* Value
Age, y				
≤ 60	1	< .0001	1	.010
60 to < 70	1.0 (0.97-1.3)		0.97 (0.7-1.3)	
70-80	2.3 (1.8-2.9)		1.4 (1.1-1.9)	
> 80	3.7 (3.0-4.8)		1.7 (1.1-2.4)	
Gender				
Male	1	.49	1	.43
Female	1.05 (0.91-1.2)		0.9 (0.8-1.1)	
TNM				
IIIa	1	< .0001	1	< .0001
IIIb	1.5 (1.1-2.0)		2.3 (1.4-3.8)	
IIIc	3.0 (2.2-4.0)		5.3 (3.2-8.6)	
Grade				
Well/moderately differentiated	1	< .0001	1	< .0001
Poorly differentiated	1.8 (1.6-2.2)		1.6 (1.3-2.0)	
Primary tumor location				
Right	1	< .0001	1	.0008
Left	0.65 (0.56-0.75)		0.7 (0.57-0.86)	
Charlson comorbidity index				
0	1	< .0001	1	.0004
1-3	1.3 (1.1-1.6)		0.8 (0.6-1.1)	
4 or more	2.9 (2.3-3.7)		1.8 (1.22-2.6)	
Year diagnosed				
2009	1	.79	1	.83
2010-2013	0.98 (0.83-1.2)		1.0 (0.9-1.1)	
Adjuvant chemotherapy				
Fluoropyrimidine only	1	< .0001	1	< .0001
Oxaliplatin doublet	0.50 (0.41-0.61)		0.54 (0.43-0.70)	

Abbreviations: CI = confidence interval; HR = hazard ratio.

for stage III colon cancer in a "real world population" of patients older than 70 years.

Patients and Methods

Patient Cohort

The New South Wales (NSW) clinical cancer registry contains demographic and clinical data for patients diagnosed or treated for cancer in NSW, covering approximately 30% of the Australian population. Data is collected from pathologic laboratories, hospitals, and oncology departments under mandatory notification of new cancer cases. We included all patients ≥ 18 years with colon cancer as per the third edition of the International Classification of Diseases for Oncology (ICD-O-3).[17] We identified 2220 patients with stage III colon cancer with complete files including adjuvant chemotherapy details. Fifty-six patients were excluded owing to death within 30 days of surgery (n = 23) or delay starting chemotherapy past 120 days (n = 33) (final sample, n = 2164).

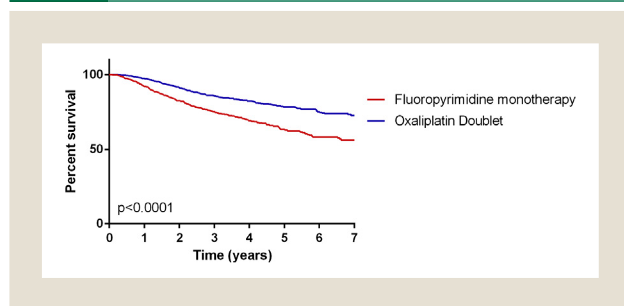
Date of death was obtained with linkage to the NSW registry of Births, Deaths, and Marriages (BDM) by the Centre for Health Record Linkage (CHeReL).[18] The censor date for survival data was December 1, 2014.

Comorbidity data and admissions during chemotherapy were obtained by CHeReL linkage of the clinical cancer registry data to the Admitted Patient Data Collection (APDC). The APDC contains all admitted patient services provided by NSW Public Hospitals, Public Multi-Purpose Services, Private Hospitals, and Private Day Procedures Centers. Comorbidities of each patient were quantified using the Charlson Comorbidity Index (CCI), which predicts mortality from a range of 22 comorbid conditions.[16] ICD-10 codes were extracted from admissions prior to diagnosis, then translated into a CCI (modified for cancer) using methods previously described.[19,20]

For quantification of chemotherapy complications, all admissions for 12 months following initiation of chemotherapy were included apart from admissions for vascular implantation, chemotherapy, routine surgery follow-up, and dialysis (ICD-10 codes Z45.2, Z51, Z48.815, and Z49, respectively). Admissions for febrile neutropenia were identified using neutropenia (ICD-10 D70) with fever and/or sepsis (ICD-10 R50.8, R50.9, A419) and/or infection (ICD-10 Chapter A, B) as previously described for Australian patients.[21]

Linkage and use of the data from the NSW clinical cancer registry, the NSW registry of BDM, and APDC was approved by

Figure 1 All-Cause Mortality by Adjuvant Chemotherapy Regimen for all Patients (n = 1626)



the NSW Population and Health Services Research Ethics Committee (approval HREC/13/CIPHS/39).

Statistical Analysis

Our primary outcome was all-cause mortality, on the basis of dates recorded in the cancer registry and BDM databases. Median values for OS and corresponding 95% confidence interval (CI) were calculated using Kaplan-Meier methods. To determine the impact of age, 2 separate Cox proportional hazard models were used to compare the effect of combination chemotherapy regimens on OS for patients ≥ 70 years and < 70 years. This age cut-off was used for consistency with previous publications and international guidelines. The following variables were included in the multivariate model: age, gender, CCI, TNM stage, primary tumor location (defined as right-sided [cecum to transverse colon] or left-sided [splenic flexure to rectosigmoid]), year of diagnosis, grade, and adjuvant treatment.

Our secondary objectives were complications of adjuvant chemotherapy by age group, as measured by number and length of admissions for 12 months after starting treatment, 12-month landmark mortality, and treatment discontinuation rate by chemotherapy regimen. The number of admissions was compared using the χ^2 test, and the mean duration of each admission by the *t* test. All statistical analyses were performed using SAS 9.2 software (SAS Institute, Inc, Cary, NC).

Results

Patient Characteristics (n = 2164) and Impact of Age on Receipt of Chemotherapy

The characteristics of patients are summarized in Table 1. Approximately one-half (49.9%) of the patients were ≥ 70 years. Patients ≥ 70 years were more likely to have right-sided primary tumors (54.6% vs. 42.7%) and poorly differentiated histology (24.3% vs. 18.3%), but despite these higher risk features, were less likely to receive adjuvant chemotherapy (60.7% vs. 89.6%) or oxaliplatin doublet chemotherapy (18.8% vs. 71.2%). Patients ≥ 80 years (n = 371) were even less likely to receive chemotherapy; only 29.4% received adjuvant fluoropyrimidine monotherapy and 3.0% received oxaliplatin doublet. Increasing TNM stage was significantly associated with receipt of oxaliplatin chemotherapy in patients < 70 years (P = .0006) but not in those ≥ 70 years (P = .08).

Patients ≥ 70 years were more likely to have a higher CCI than younger patients (39.4% vs. 23.3% with CCI > 0). Increasing CCI was associated with decreased administration of adjuvant chemotherapy and oxaliplatin doublet treatments in all patients (P < .001), patients < 70 years (P = .04), and patients ≥ 70 years (P < .0001).

Although the majority of patients on fluoropyrimidine monotherapy received oral capecitabine rather than intravenous fluorouracil (83.9% vs. 16.1%), only a minority of patients treated with oxaliplatin doublet chemotherapy had oral capecitabine (CAPOX) rather than intravenous fluorouracil (FOLFOX) (13.7% vs. 86.3%). There was a similar pattern of use in patients ≥ 70 years and < 70 years.

Complications of Chemotherapy

Chemotherapy complications were quantified with hospital admissions for 12 months following initiation of adjuvant chemotherapy and 12-month landmark mortality.

In patients who received fluoropyrimidine monotherapy, there was no significant difference in proportion of patients < 70 years admitted to hospital compared with those ≥ 70 years (49.7% vs. 49.8%; P = .59) or the mean duration of admissions (5.92 vs. 5.59 days; P = .66). In contrast, patients ≥ 70 years who received oxaliplatin were more likely to be admitted to hospital (67.0% vs. 53.5%; P = .0006) and require multiple admissions (37.4% required ≥ 2 admissions vs. 25.5%; P = .0008) than younger patients on oxaliplatin. There was a nonsignificant trend to longer admissions (mean length of admission, 6.1 vs. 4.8 days; P = .09).

In patients ≥ 70 years, those treated with oxaliplatin were more likely to be admitted to hospital (67.0% vs. 49.6%; P < .0001) and require multiple admissions (37.4% ≥ 2 admissions vs. 26.1%; P = .003) than those on fluoropyrimidine monotherapy. Although there was no significant difference in admissions for febrile neutropenia between age groups for patients on oxaliplatin (6.9% vs. 4.7%; P = .19), patients ≥ 70 years on oxaliplatin were more likely to be admitted for febrile neutropenia than those on fluoropyrimidine monotherapy (6.9% vs. 1.8%; P = .0008).

Patients ≥ 70 years were also less likely to complete adjuvant oxaliplatin doublet chemotherapy than those < 70 years, defined as receiving < 3 months of treatment (18.7% vs. 7.6%; P < .0001). There was no difference in completion rates between age groups for patients on fluoropyrimidine alone (P = .33).

Patients ≥ 70 years who received adjuvant oxaliplatin doublet chemotherapy had a significantly poorer 12-month landmark OS than younger patients (5.9% vs. 1.7%; P = .0006). This difference between age groups was not seen in patients who received fluoropyrimidine monotherapy (8.6% vs. 4.5%; P = .06). Within patients ≥ 70 years, there was no significant difference in 12-month OS between those who received fluoropyrimidine monotherapy compared with those who received oxaliplatin doublet (8.6% vs. 5.9%; P = .23).

Efficacy of Adjuvant Chemotherapy in the Elderly

In all patients who received adjuvant chemotherapy (n = 1626), oxaliplatin doublet chemotherapy improved OS compared with fluoropyrimidine alone (multivariate hazard ratio [HR], 0.54; 95% CI, 0.43-0.70; P < .0001) (Table 2, Figure 1). Increasing age, comorbidity score, TNM stage, poorly differentiated grade, and right-sided primary tumor location were all significantly associated with poorer OS in both univariate and multivariate analyses. Use of

Table 3 Univariate and Multivariate Analyses for Overall Survival Stratified by Age

Characteristic	Patients < 70 Years (n = 971)			Patients ≥ 70 Years (n = 655)		
	Univariate HR (95% CI)	Multivariate (95% CI)	*P* Value	Univariate HR (95% CI)	Multivariate	*P* Value
TNM						
IIIa	1	1	< .0001	1	1	< .0001
IIIb	1.6 (0.9– 2.9)	1.7 (0.9-3.3)		1.5 (1.04- 2.0)	3.0 (1.4-6.4)	
IIIc	4.1 (2.3-7.1)	4.4 (2.3-8.2)		2.7 (1.9-3.9)	6.4 (3.0-13.9)	
Grade						
Well/moderately differentiated	1	1	.0007	1	1	.01
Poorly differentiated	2.0 (1.5-2.7)	1.7 (1.3-2.5)		1.6 (1.4-2.0)	1.5 (1.1-2.0)	
Primary tumor location						
Right	1	1	< .0001	1	1	.39
Left	0.6 (0.5-0.8)	0.5 (0.4-0.7)		0.80 (0.6-0.9)	0.9 (0.7-1.2)	
Charlson comorbidity index						
0	1	1	.01	1	1	.01
1-3	0.8 (0.6-1.2)	0.6 (0.4-1.0)		1.2 (1.01-1.5)	0.9 (0.6-1.2)	
4 or more	2.4 (1.4-3.9)	1.8 (0.9-3.3)		2.4 (1.8-3.2)	1.9 (1.2-3.1)	
Adjuvant chemotherapy regimen						
Fluoropyrimidine only	1	1	< .0001	1	1	.005
Oxaliplatin doublet	0.6 (0.4-0.8)	0.4 (0.3-0.6)		0.7 (0.5-0.9)	0.6 (0.5-0.8)	

Abbreviations: CI = confidence interval; HR = hazard ratio.

capecitabine, rather than 5-fluorouracil, was not significantly associated with OS in either the fluoropyrimidine monotherapy ($P = .82$) or oxaliplatin doublet ($P = .48$) treatment groups.

When stratified by age, the addition of adjuvant oxaliplatin demonstrated an OS benefit in patients < 70 years (HR, 0.56; 95% CI, 0.41-0.77; $P = .0003$) and ≥ 70 years (HR, 0.72; 95% CI, 0.53-0.98; $P = .037$), which remained significant in multivariate analysis (Table 3, Figure 2). Gender and year of diagnosis were not significant in univariate analysis and were therefore not included in the final model.

Oxaliplatin doublet chemotherapy was associated with a preserved OS benefit in patients with significant comorbidity (patients with CCI ≥ 2, univariate HR, 0.40; 95% CI, 0.29-0.61; $P < .0001$), including patients < 70 years (HR, 0.38; 95% CI, 0.16-0.94; $P = .02$), but not in patients ≥ 70 years (HR, 0.67; $P = .28$). Exploratory subgroup analysis demonstrated a diminishing OS benefit to oxaliplatin with increasing age, with the HR approaching 1 (no benefit) for more elderly patients (Figure 3).

Discussion

Colon cancer is more common in the elderly, because 67 is the median age at diagnosis, and almost 40% of patients are ≥ 75 years old.[3,4] Despite this, the pivotal phase III trials that demonstrated the improved OS with adjuvant oxaliplatin chemotherapy in stage III colon cancer included a only small minority of patients older than 70 years, and consequently were unable to show a benefit to oxaliplatin in this population.[3,22]

The principle finding of the current study is a statistically significant improved OS with adjuvant oxaliplatin doublet chemotherapy compared with fluoropyrimidine monotherapy in patients ≥ 70 years with stage III colon cancer (HR, 0.72; $P = .037$). This difference remained significant in multivariate analysis, which included a comorbidity index (multivariate HR, 0.64; $P = .005$).

There is no consensus in the literature regarding the benefit of adjuvant oxaliplatin for elderly patients. Neither DFS nor OS was significantly improved in the small minority of elderly patients in the MOSAIC or NSABP-07 trials, or in the ACCENT database.[6,7,10] In contrast, pooled individual patient data from 4 other randomized trials, which included comorbidities as a covariate, demonstrated improved DFS and OS with oxaliplatin.[11] Similarly, there are disparate results seen in "real-world" patient series. Analysis of the SEER database showed a persistent benefit to adjuvant oxaliplatin in patients > 70, although with inconsistent results in patients older than 75, and in those with significant comorbidities.[12,13] Other results from smaller retrospective series are conflicting.[14,23] One common criticism of all the above studies, as well as the current work, is the omission of an assessment for medical frailty, an important and distinct entity to comorbidity.[24]

Figure 2 All-Cause Mortality by Adjuvant Chemotherapy Regimen Stratified by Patient Age at Diagnosis

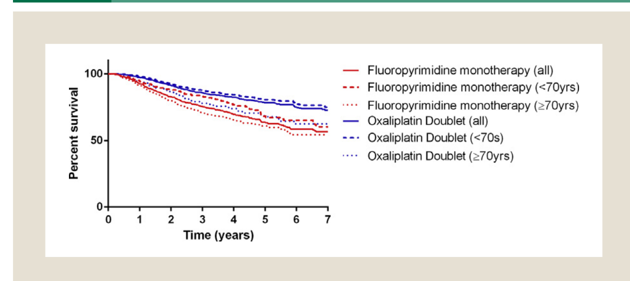
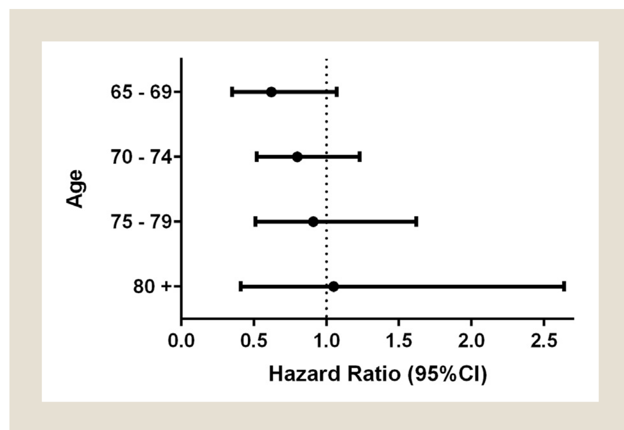


Figure 3 Unadjusted Hazard Ratio for Overall Survival Benefit to Oxaliplatin Chemotherapy Stratified by Age. The Circle on Each Bar Represents the Hazard Ratio for That Age Group, and the Bar Shows the 95% Confidence Interval



Abbreviation: CI = confidence interval.

It is important to highlight that the elderly patients who received oxaliplatin chemotherapy in the current study are likely to represent a highly selected subgroup. This is supported by the observed high completion rate of adjuvant oxaliplatin doublet chemotherapy in patients $\geq$ 70 years, although we note that data regarding chemotherapy dosing, dose reductions, and delays, which may provide further insight, are not available. Consistent with other published series, we found increasing age was associated with decreased receipt of any adjuvant chemotherapy and oxaliplatin doublet chemotherapy, with only 18.8% of patients $\geq$ 70 years, and 3% $\geq$ 80 years, receiving oxaliplatin.[13,14,25] Similarly, increasing level of comorbidity, quantified by the CCI, was also associated with decreased receipt of oxaliplatin ($P < .0001$), with most of the elderly patients who received oxaliplatin (86.2%) having minimal comorbidities (CCI < 2).

Despite presumed patient selection for oxaliplatin doublet therapy, hospital admissions were modestly increased in elderly patients compared with younger patients. Elderly patients who received oxaliplatin doublet chemotherapy were more likely to be admitted to hospital, require multiple admissions to hospital, or require admissions for febrile neutropenia. These observations were not seen in patients receiving fluoropyrimidine monotherapy, consistent with current literature that shows increased toxicity in the elderly from doublet chemotherapy, but not fluoropyriminde monotherapy.[9,11,14,26,27] We acknowledge that the hospital admission data do not reflect all toxicity, as at least some complications are likely to have been managed out of hospital. It also important to highlight we did not find a significant difference in 12-month landmark OS between chemotherapy regimens in elderly patients.

The decision to proceed with adjuvant chemotherapy in an elderly patient is complicated and depends on many patient health and social factors. The patient's age, comorbidities, and perceived minimal benefit are the predominant reasons for withholding adjuvant chemotherapy in elderly patients.[28] Although the average life expectancy of an otherwise healthy 70-year-old male and female is approximately 8 years and 14 years, respectively,[5] many elderly patients have significant comorbidities that could shorten survival. Although we used the 70-year age cut-off in our primary analysis for consistency with other publications, our exploratory subgroup analyses demonstrated, as expected, a diminishing benefit to adjuvant oxaliplatin with increasing age. Moreover, although the benefit for adjuvant oxaliplatin increases with time in younger patients, in older patients it decreases, so by 3 years after surgery, the competing mortality risks eliminate the benefit of doublet adjuvant chemotherapy.[10] The recently presented data from the IDEA collaboration supports a risk-adapted approach to duration of adjuvant chemotherapy, with a shorter duration of adjuvant chemotherapy in lower risk disease to reduce treatment-associated toxicities.[29] These data, along with our study, support the role of an individualized treatment approach, rather than strict age cut-offs, when determining the optimal adjuvant strategy for elderly patients.

There are limitations to the current study. First, we analyzed an observational database and acknowledge important unmeasured confounders and selection bias between treatment groups. Second, there was no data available regarding chemotherapy dosing, dose reductions, or treatment delays, for any patients. However, although it is likely that most of the elderly patients received dose modifications to improve tolerability,[30] we still found an OS benefit.

Conclusion

Our study demonstrates a survival benefit to adjuvant chemotherapy with an oxaliplatin doublet over fluoropyrimidine alone for patients $\geq$ 70 years with stage III colon cancer. However, we also found evidence of modestly increased hospital admission rates with doublet treatment. The potential for survival benefit must be weighed against the increased risk of toxicities in this population, as well as individual patient life-expectancies, based on comorbidities and other factors.

Clinical Practice Points

- The optimum adjuvant chemotherapy regimen for elderly patients with stage III colon cancer is unknown, with conflicting results from clinical trials and population studies.
- Data from an Australian cancer registry (n = 2164) was interrogated to examine the utilization, safety, and efficacy of adjuvant chemotherapy in elderly patients with stage III colon cancer.
- Patients $\geq$ 70 years are less likely to receive any adjuvant chemotherapy than younger patients (61% vs. 90%) or oxaliplatin doublet adjuvant chemotherapy (19% vs. 71%).
- Addition of oxaliplatin to fluoropyrimidine as adjuvant chemotherapy improves survival in patients $\geq$ 70 years (HR, 0.64; $P = .005$). This benefit remained significant in the multivariate analysis after adjusting for age, comorbidity, TNM stage, grade, and primary tumor location (multivariate HR, 0.72; $P = .037$). The survival benefit appears to reduce with increasing age.
- Patients $\geq$ 70 years who received oxaliplatin chemotherapy are more likely to be admitted to the hospital or cease treatment early, reflecting increased toxicity in this population.
- Adjuvant oxaliplatin should be considered in elderly patients with stage III colon cancer. Rather than use strict age cut-offs, we recommend a comprehensive geriatric assessment of elderly

patients, followed by a detailed discussion of the risks and benefits of adjuvant treatment, to permit optimal individualization of treatment for each patient.

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Disclosure

The authors have stated that they have no conflicts of interest.

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