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Reduction of plasma fibulin-1 predicts poor survival in metastatic colorectal cancer

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Bachelor of Medical Science (Honours), University of Sydney

A thesis submitted in fulfilment of the requirements for the degree of Masters of Philosophy

Discipline of Pharmacology, School of Medical Science
University of Sydney, Australia
February 2015
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Abstract

In Australia, colorectal cancer (CRC) is the second most common diagnosed cancer and the second leading cause of cancer-related deaths. Patients diagnosed with metastatic CRC (mCRC) have a 5-year survival rate of 10%; a drastic reduction from 90% in patients with localised CRC. For patients with mCRC, palliative chemotherapy is the first-line of treatment. Approximately 50% of patients will respond to chemotherapy; however, discriminating which patients will respond to therapy prior to starting remains unknown. Therefore, biomarkers are required to predict patient outcomes to aid treatment selection and improve patient survival. Systemic inflammation and extracellular matrix (ECM) remodelling are vital factors in carcinogenesis; mCRC patients with poorer survival tend to have elevated systemic inflammation and excessive ECM remodelling. Fibulin-1 is an ECM protein and plasma glycoprotein which interacts with many ECM proteins to regulate numerous aspects of carcinogenesis.

The aim of this research project was to evaluate the prognostic value of fibulin-1 expression in patients with mCRC and investigate the role of fibulin-1 in CRC carcinogenesis.

To evaluate the prognostic value of fibulin-1 in mCRC, fibulin-1 expression in clinical samples and cell lines were investigated with a variety of techniques, including immunohistochemistry, selective reaction monitoring mass spectrometry, Western blotting and qualitative real-time PCR. The functional role of fibulin-1 on migration and angiogenesis was assessed with wound scratch assay and HUVEC tube formation assay, respectively.

A reduction of plasma fibulin-1 was associated with elevated systemic inflammation and poorer survival. Differential expression of fibulin-1 was observed in human colonic fibroblast and CRC cell lines. I was unable to identify differences in migration and angiogenesis according to basal secreted fibulin-1 expression in CRC cell lines. In conclusion, plasma levels of fibulin-1 may serve as a novel prognostic biomarker.
Declaration

I certify that the work in this thesis entitled “Reduction in plasma fibulin-1 predicts poor survival in metastatic colorectal cancer” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than the University of Sydney.

I also certify that the thesis is an original piece of research and has been written by me. Any assistance that I have been provided in my research project has been appropriately acknowledged.

The research presented in this thesis was approved by the Sydney Southwest Area Health Service Research Ethics Committee (reference number: #CH62/6/2006-132); and financially supported by funding from a Cancer Institute NSW Career Development Fellowship (K.A.C, #10/CDF/2-36).

Thien Phuoc Huynh

Student number: 306170949

February 2015
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Firstly, I would like to sincerely thank Kellie Charles for her endless patience, supervision and knowledge. You have my best interest at heart, and have always encouraged me to realise my full potential and passion for research. All your advice and support has shaped me into the researcher I am today, and your push and drive motivate me to achieve that extra 110% in everything I do. Thank you to my lab mates – past and present – especially Chloe Lei, Vid Perera, Ben Harris and Diana Shinko for all that you have done to make each day in the lab fun. Additionally, I would like to thank Matthew McKay and Sarah Hayes for being my mass spectrometry guru; all your tips and tricks helped me immensely when I had no idea what I was doing or when the machines played up.

Special thanks to the Transplant guys Mou, Miriam, Mamdouh and Eithne for always finding excuses to enjoy cake, caffeine and booze, bringing joy and laughter to random situations, and for being such a supportive cheer squad! You guys are seriously crazy! To all my friends and staff in Pharmacology, in particular, Cho Zin, Joe, Amanda, Rosie, Thomas, Chi, Jiesi, Isla, Athena, Silvana, Cheryl and Keith, thanks for all the advice and support you all have provided and willingness to being guinea pigs for my baked goods over the years.

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I am forever grateful for the sacrifices my parents have made to allow me to have a bright and successful future, and for their support despite not completely understanding what my project entails. My sisters for being my sisters, even though you all drive me insane sometimes. Also, to my in-laws, for listening to the joys and dramas of my life.

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Contributions

For this thesis, colorectal cancer patient plasma samples were obtained by Dr Wei Chua and Prof Stephen Clarke from the Department of Medical Oncology, Concord Repatriation General Hospital, Sydney. Top-14 plasma protein immunodepletion was carried out by Ms Janniche Torsvik at the Australian Proteome Analysis Facility, Sydney. ELISA for IL-6, IL-8 and CXCL1 were conducted by Ms Chloe Lei from the Cancer Therapeutics Research Group at University of Sydney. Assistance with aspects of selective reaction monitoring mass spectrometry (SRM-MS) assay development and quantitation was provided by Dr Matthew McKay and Dr Sarah Hayes (nee Randall), from the Australian Proteome Analysis Facility, Sydney. Surgical resection of CRC tumour samples were performed by Prof Pierre Chapuis and his team from the Department of Colorectal Surgery, Concord Repatriation General Hospital, Sydney; and histology sections for immunohistochemistry (IHC) staining were sectioned by Ms Candice Clarke from the Department of Anatomical Pathology, Concord Repatriation General Hospital, Sydney. Human idiopathic pulmonary fibrosis lung parenchymal tissue obtained from resected human lungs and post mortem organ donors from St Vincent’s Hospital, Sydney, was kindly provided by Assoc Prof Janette Burgess and Mr Gavin Tjin (Woolcock Institute of Medical Research, Sydney) to use as positive controls in the IHC experiments. I conducted all the other work in this study, including: sample processing, developing and optimising SRM-MS methods, SRM-MS analysis and quantitation, Western blotting, IHC, and all the in vitro experiments.

The proteomics work was undertaken at the Australian Proteomics Analysis Facility (APAF) at Macquaire University, the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS) program. I would also like to acknowledge the clinical nurses and patients at Concord Repatriation General Hospital and Royal Prince Alfred Hospital for their time and effort.
Presentations and awards

Oral presentations


Poster presentations


**Awards**

Australian Postgraduate Award Scholarship with Rotary Health Top-up: Feb 2011 – Aug 2014
### Common abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ACPS</td>
<td>Australian Clinico-Pathological Staging</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADAMTS-1</td>
<td>A disintegrin and metallopeptidase with thrombospondin motif 1</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BRAF</td>
<td>Proto-oncogene B-raf</td>
</tr>
<tr>
<td>CAFs</td>
<td>Cancer-associated fibroblasts</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of differentiation 3; a T cell co-receptor</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8; a T cell co-receptor found on cytotoxic T cells</td>
</tr>
<tr>
<td>CD44</td>
<td>Receptor for hyaluronic acid to mediate cell-cell and cell-matrix interactions</td>
</tr>
<tr>
<td>CD133</td>
<td>A marker of cancer stem cells</td>
</tr>
<tr>
<td>CD45RO</td>
<td>A marker of activated and memory T cells</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
</tr>
<tr>
<td>cps</td>
<td>Count per second</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Chemokine (C-X-C motif) receptor 2</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FBLN1</td>
<td>Fibulin-1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FOLFOX</td>
<td>Combination of 5-FU, leucovorin and oxaliplatin chemotherapy</td>
</tr>
<tr>
<td>FOLFIRI</td>
<td>Combination of 5-FU, leucovorin and irinotecan chemotherapy</td>
</tr>
<tr>
<td>GMS</td>
<td>Glasgow Microenvironment Score</td>
</tr>
<tr>
<td>GPS</td>
<td>Glasgow Prognostic Score</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KM</td>
<td>Klintrup-Makinen</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mCRC</td>
<td>Metastatic colorectal cancer</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MMTS</td>
<td>Methylmethanethiosulfonate</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Neutrophil-lymphocyte ratio</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBS</td>
<td>Pharmaceutical Benefit Scheme</td>
</tr>
<tr>
<td>PFS</td>
<td>Progressive-free survival</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
</tr>
<tr>
<td>PLR</td>
<td>Platelet-lymphocyte ratio</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RAF</td>
<td>Oncogene which is an acronym for “Rapidly Accelerated Fibrosarcoma”</td>
</tr>
<tr>
<td>RAS</td>
<td>Oncogene which is an acronym for “Rat sarcoma”</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria In Solid Tumors</td>
</tr>
<tr>
<td>RPL32</td>
<td>60S ribosomal protein L32</td>
</tr>
<tr>
<td>SAA1</td>
<td>Serum amyloid A1</td>
</tr>
<tr>
<td>SRM-MS</td>
<td>Selective reaction monitoring mass spectrometry</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEAB</td>
<td>Triethylammonium bicarbonate</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of MMP</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XIC</td>
<td>Extracted ion chromatogram</td>
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Chapter 1
Introduction
1.1 Colorectal cancer (CRC)

1.1.1 Global overview

Colorectal cancer (CRC) is the third most common cancer and a leading cause of cancer-related deaths worldwide. With over 1.3 million new cases diagnosed and approximately 700,000 deaths reported each year, it accounts for 9.7% and 8.5% of the total cancer cases and deaths worldwide, respectively (1). At the end of 2012, over 3.5 million patients were still alive after 5 years of being diagnosed with CRC, making it the second most prevalent cancer worldwide after breast cancer (2). The incidence of CRC affects men and women almost equally and there is wide geographical variation in the distribution of CRC throughout the world; approximately 55% of the cases occur in more developed regions, with the highest estimated age-standardised incidence rates are observed in Australia/New Zealand (1). Improvements in CRC screening tests and treatment options have greatly reduced mortality rates, although this rate is dependent on availability of the tests and treatments.

1.1.2 CRC in Australia

In Australia, CRC is the second most common cancer diagnosed among men and women and the second leading cause of cancer-related deaths. In 2012, 15,840 new cases were estimated and 3,982 deaths reported for CRC (3). CRC incidence and mortality is associated with increasing age from around 50 years, with the current median age of patients being 69.3 years (3). More than 90% of colorectal cancer cases occur in people aged 50 or older; and the current risk of being diagnosed with CRC by the age of 85 is 1 in 12 and the risk of dying from CRC before the age of 85 is 1 in 46 (4). Between 1991 and 2010, incidence rates have remained stable whilst mortality rates have declined, for men this rate fell by 41% (from 34 to 20 per 100,000) and for women, 45% (from 24 to 13 per 100,000) (3). The 5-year survival rate has improved in recent years, from 48.0% to 66.2% during 1982 – 1987 to 2006 – 2010 (5). This decline in mortality and improvement in survival could be attributed to improved national
screening programs, diagnostic techniques and treatment options, which have assisted in the early detection of pre-malignant polyps and the extension of patient survival.

The financial burden for caring for Australian CRC patients is large. In 2008 – 2009, the Australian government’s total expenditure for CRC was $459.85 million and was considered as the highest of the total expenditure for cancer and other neoplasms; a small portion (7%) of the cost funded the National Bowel Cancer Screening Program and the remaining cost is contributed to subsidise treatment and hospitalisation fees for patients (6). The cost to the health system for treating a CRC patient varies from less than $2,000 to detect and remove a non-malignant polyp to up to approximately $66,000 to completely treat metastatic CRC (mCRC) patients (7). With the subsidy of first-line standard chemotherapy agents such oxaliplatin, irinotecan and bevacizumab under the Pharmaceutical Benefit Scheme (PBS) in treating patients with mCRC, from 2009 – 2010, the PBS cost for these drugs totalled $70 million. The monoclonal antibody bevacizumab alone accounted for $65.4 million (93% of the PBS payments) which contributes to an approximate 50% increase in the chemotherapy cost, making it one of the most high-cost medicines subsidised by the PBS in 2009 (8, 9). As a result of this increase in chemotherapy cost to treat mCRC patients, an understanding in CRC biology to predict for patient outcomes and response to chemotherapy is of high priority to extend the well-being of CRC patients and reduce the financial burden associated with CRC.

1.2 Development and staging of CRC

CRC is defined as the cancers confined by the boundaries of the colon, rectum and anal canal (10). When normal colon cells grow uncontrollably from the colon wall and into the lumen, they result in polyps and over time, these polyps may develop into benign adenomatous polyps (adenomas). Histologically, adenomas can be classified as tubular, tubulovillous, villous or serrated structures (11). Most CRCs arise from adenomas; the likelihood of these adenomas developing into CRC can be assessed by their histology and size; villous adenomas have a
higher malignancy potential and adenomas greater than 1 cm are likely to give rise to CRC (11). It is approximated that 5% of adenomas become malignant, a process which takes up to 5 – 10 years (12) and is a result of a series of genetic alterations and mutations (13). These malignant adenomas originating in the colon wall are confined locally before invading through the colon wall to spread to neighbouring lymph nodes and distant organs (13). Early detection and removal of these adenomas are likely to decrease the incidence and mortality of CRC. The risk of developing CRC is associated with increased age, personal history of adenomas or inflammatory bowel disease, family history of adenomas or CRC, and inherited disorders which lead to increased development of polyps such as familial adenomatous polyposis, and hereditary non-polypsis colon cancer (Lynch syndrome) (14).

In Australia, the most common CRC staging systems are TNM and Australian Clinico-Pathological Staging (ACPS) system. The TNM system, developed by the American Joint Committee on Cancer, provides information on the tumour (T), lymph nodes (N) and metastasis (M) (10). T indicates the size of the cancer and the extent to which the cancer penetrates through the colon and rectum wall, N describes the extent of neighbouring lymph node involvement and M indicates whether the cancer has spread to distant lymph nodes and organs. Once the T, N and M categories have been determined, their scores are combined and the overall stage is assigned; from stage I (less advanced) to IV (most advanced) (Figure). The ACPS system is derived from the original Duke’s staging criteria to assist clinicians in accurately determining the prognosis and treatment for patients (15, 16). This system is more advantageous than the Duke’s system as it utilises all information available (such as clinical, radiologic, operative, and pathologic); whereas the Duke’s system was entirely based on the use of pathological examination of the resected tumour and does not account for distant metastases or residual cancer post-resection by the surgeon (15). Early stages (A) of CRC are localised within the colon and rectum wall, whereas invasion through the serosal surface is
classified as Stage B. Advanced stages of CRC involve the spread of malignant cells to neighbouring lymph nodes (Stage C) and distant organs and lymph nodes (Stage D) (Figure 1).

![Figure 1](image.png)

**Figure 1.** Stages of CRC defined by TNM and ACPS system. Schematic of the development of CRC from localised tumour (Stages I and II, according to TNM; or Stages A and B according to ACPS) through the mucosal wall to spread to neighbouring lymph nodes (Stage III and C) and distant organs (Stage IV and D). Image adapted from: Davis & Newland. ANZ Journal of Surgery. 1983; 53(3): 211 – 221 and 7th edition of the AJCC Cancer Staging Manual (2010) (10, 16).

Patient survival is highly dependent on the stage of CRC at diagnosis, with the 5-year survival rate for patients with localised, early stages of CRC approximately 90%; in patients with increasing invasion and metastasis towards neighbouring lymph nodes this rate drops to 70% and further drops to 10% once the cancer has spread to distant organs (14). One of the main reasons for these poor outcomes is that CRC is a heterogeneous disease. Genetic and molecular features of the cancer, as well as the host response to the cancer, determine the large inter-individual differences in prognosis and response to therapy (17).

### 1.3 Treatment for CRC patients

The stage of CRC at diagnosis also plays an important role in determining treatment options for patients. When localised to the walls of the bowel (Stage I and II), CRC is treated, and often
cured, with surgery alone. Once the cancer spreads to distant lymph nodes (Stage III), adjuvant radiotherapy or chemotherapy is offered with surgery to prevent tumour recurrence. Patients with mCRC (Stage IV) have the cancer metastasising to distant organs and surgery is unlikely to cure the disease; palliative chemotherapy regimens are given to these patients to improve their quality of life and prolong their survival (18).

In this study, we have only analysed samples in patients with Stage IV CRC who have received first-line palliative chemotherapy.

### 1.3.1 Cytotoxic chemotherapy

First-line chemotherapy in mCRC is based on 5-fluorouracil (5-FU) (or capecitabine, an oral pro-drug of 5-FU), in combination with leucovorin (folinic acid) (19). 5-FU is utilised in the treatment of CRC to impede RNA and DNA synthesis of rapidly dividing cancer cells, and to inhibit thymidylate synthase to further disrupt DNA synthesis and repair. Leucovorin stabilises the binding of 5-FU to thymidylate synthase to further enhance its inhibitory effect on thymidylate synthase (20). The introduction of oxaliplatin, a platinum-base DNA alkylating agent that exerts its cytotoxic effects by forming inter- and intra-strand DNA adducts; and irinotecan, a topoisomerase I inhibitor, to 5-FU/leucovorin for chemotherapy regimens FOLFOX and FOLFIRI, respectively, have provided additional cytotoxic effect by preventing DNA replication. These two regimens significantly extend the median overall survival (OS) of patients with mCRC to 15 – 20 months, in comparison to approximately 12 months amongst patients treated with 5-FU and leucovorin (21-24). In a study conducted by Tournigand et al., both FOLFOX and FOLFIRI regimens achieve similar response rates (54% vs. 56%, respectively) and median OS outcomes (20.6 months vs. 21.5 months, respectively); however, as expected both these regimens differed in toxicity profiles (detailed in Section 1.3.3) (24).
1.3.2 Targeted therapy

Within the last decade, the development of novel pharmacological agents targeting molecular pathways known to play a vital role in carcinogenesis has shown significant improvement to the clinical outcome of mCRC patients. Vascular endothelial growth factor (VEGF) is a crucial mediator in angiogenesis. Upon binding to its associated receptor on endothelial cells, VEGF activates cascades of signalling pathways to promote the growth and migration of endothelial cells to form new blood vessels to supply oxygen, growth factors and cytokines to the tumour and allow it to grow and spread successfully (25). Bevacizumab is a recombinant humanized monoclonal antibody which targets VEGF to inhibit the growth of tumours by preventing the formation of new blood vessels being supplied to the tumour (26). Epidermal growth factor receptor (EGFR) is another critical pathway involved in promoting the proliferation of malignant cells during carcinogenesis. Upon activation by its ligands, such as EGF and transforming growth factor-α on malignant cells, activation of two signalling pathways (RAS-RAF-MAPK for proliferation and PI3K-Akt/mTOR for survival) facilitate the growth of tumours (27). Cetuximab, a chimeric monoclonal antibody, and panitumumab, a fully human monoclonal antibody, are two monoclonal antibodies targeting EGFR to reduce or delay the growth of cancer (28, 29). These monoclonal antibodies extend the median OS to 20 – 30 months when used in combination with first-line FOLFOX or FOLFIRI chemotherapy regimens (30-35). As such, these combination drug regimens are the standard of care for mCRC patients starting chemotherapy.

1.3.3 Toxicities

Despite improvements in extending patient survival with the chemotherapy treatment options mentioned previously, not all mCRC patients benefit or tolerate chemotherapy in the same manner. The balancing act in the administration of chemotherapy treatment is finding the optimal dose required to deliver the highest efficacy of the drug to eradicate the tumour whilst minimising unwanted toxicities. Patients who experience severe toxicities require dose delays
and/or reduction, which potentially impacts on tumour response towards chemotherapy and reduces the efficacy of the chemotherapy.

One of the main problems with the use of cytotoxic chemotherapy drugs is the non-selective nature of targeting highly proliferating cells. In addition to killing highly proliferative tumour cells, chemotherapy drugs also target rapidly proliferating healthy cells in the bone marrow, mouth, gastrointestinal tract and hair follicles, which can lead to the common toxicities observed in patients being treated with chemotherapy, such as neutropenia, anaemia, diarrhoea and hair loss. Toxicities associated with 5-FU include mild to moderate cases of nausea, vomiting and diarrhoea, loss of appetite, mucositis and neutropenia and anaemia (21). Severe neutropenia places patients at a higher risk of being susceptible to infections which leads to delays in chemotherapy treatment, reduced dosage, prolonged hospitalisation, increase use of medical resources and reduction of quality of life, and even death (36). With the addition of oxaliplatin, patients also experience debilitating peripheral neuropathy (which may be triggered by exposure to cold temperatures) that often involves sensory changes, movement difficulties and autonomic symptoms e.g. paresthesia, hypoesthesia, dysesthesia, shortness of breath or difficulty swallowing, lack of muscle control (spasms and cramps) and changes in proprioception (37). Neuropathy can cause severe pain and the intensity of this pain can impede on the ability of patients to perform daily activities. As a result, neuropathy is a dose-limiting toxicity as patients tend to require a reduction in oxaliplatin dosage or withdrawal from treatment to minimise the incidence, duration and severity of these side effects, rendering the treatment as ineffective (37). Toxicities associated with irinotecan include severe diarrhoea and neutropenia (23, 38).

Although molecular targeted therapy provides an advantageous survival benefit for mCRC patients, these treatment options are also associated with distinct toxicity profiles. Bevacizumab has been shown to be generally safe and tolerable in mCRC patients; only to be
accompanied by a few manageable adverse effects such as severe hypertension, proteinuria and bleeding, which are common toxicities observed in all inhibitors of the VEGF signalling pathway (39-41). Over time, these adverse effects can be detrimental and lead to further complications such as cardiovascular events (cerebral infarction, transient ischemic attacks, myocardial infarction and angina), and heart and kidney damage or failure. Therefore, precautions are required prior to administering bevacizumab to patients with high blood pressure, and pre-existing cardiovascular and renal conditions, which are often observed in elderly patients. Monoclonal antibodies targeting EGFR lack many of the severe side effects commonly observed with cytotoxic 5-FU-based chemotherapy; however, they are associated with a set of unique dermatological toxicities such as acne-like rashes, itchy and dry skin, nail infections, hair abnormality and mucositis, which affect approximately 90% of patients (33, 42, 43). The most common adverse side effect observed in mCRC patients is the development of an acne-like rash which usually affects the face, scalp and upper torso (42). The severity of the rash is dose-dependent and is an indicator of the efficacy of EGFR inhibitors; however, not only will it cause physical discomfort for patients, studies have found psychological and emotional effects associated with cutaneous toxicities of EGFR inhibitors which can impact on a patient’s quality of life and likelihood to adhere to therapy (44).

1.3.4 Biomarkers for chemotherapy response

Despite vast improvements to chemotherapy regimens at extending survival, only 30 – 50 % of patients will respond and benefit from cytotoxic and targeted chemotherapy treatment (24, 39, 43, 45). Unfortunately, clinicians cannot discriminate which patients will respond or experience toxicity to these chemotherapy regimens prior to or during treatment. Due to the unpredictability of the response rates and the toxicities, a better understanding of how we might be able to predict the treatment outcomes using predictive biomarkers is required. By doing so, improvements to the treatment selection process for patient treatment could
enhance response rates, reduce a patient’s risk to experiencing toxicities and further improve survival outcomes in mCRC patients.

Extensive research has been conducted to identify and investigate putative biomarkers for predicting response to first-line 5-FU-based chemotherapies. Previously investigated biomarker candidates include enzymes and transmembrane transporters involved in the metabolism and transport of 5-FU, oxaliplatin and irinotecan, such as thymidylate synthase, dihydropyrimidine dehydrogenase, glutathione S-transferase, topoisomerase I, and ABC/SLC transporters, and components of the DNA damage repair machinery, such as excision cross-complementing and X-ray cross complementing enzymes (46, 47). However, clinical studies have generally been conducted in small cohorts, which have made the validation of biomarkers difficult. To date, there have been no robust and reliable biomarkers for the any of cytotoxic chemotherapy drugs introduced into clinical practice for treating mCRC patients.

Currently, there are no biomarkers in clinical use for predicting for bevacizumab response in mCRC patients to aid in the treatment selection process. There are limited clinical studies investigating components of the angiogenesis pathway (either VEGF -dependent or - independent), such as VEGF-A protein, VEGF receptors, carcinoembryonic antigen, CD133, EGFR, which have shown promising results as possible predictive biomarkers for bevacizumab response in mCRC patients, however further research needs to be conducted to validate these biomarker candidates (48-52).

Many studies have investigated various biomarker candidates for predicting response to EGFR monoclonal antibodies, which included markers for EGFR expression and mutations in two downstream EGFR signalling pathways; RAS-RAF-MAPK-ERK and PI3K-AKT-mTOR (46, 53). Components of these signalling pathways are proto-oncogenes which modulates DNA repair, cell proliferation, differentiation and migration, angiogenesis and invasion; and serve as an
intermediate between extracellular ligand binding to EGFR and intracellular transduction of
the signalling pathways (53). Mutations in KRAS are observed in the tumours of approximately
40% of mCRC patients and have been identified as a predictor of poor response and OS to both
cetuximab and panitumumab, in comparison to those patients without KRAS mutations in their
tumours (33, 43, 54, 55). However, 40 – 60% of patients with wild-type KRAS tumours still do
not respond to such therapy.

Upon further analysis, it has been found that patients with NRAS mutations also contribute to
anti-EGFR antibody insensitivity and poorer OS in comparison to patients with wild-type RAS
(30, 35). It has also been suggested that a proportion of these wild-type KRAS patients carry a
mutation in the BRAF gene (present in 5 – 10% of all mCRC tumour cases and are mutually
exclusive of KRAS mutations) which also affects patient response to these agents (33, 56).
Within the PI3K-AKT signalling pathway, PIK3CA is activated through EGFR signalling and PTEN
is an inhibitor of PIK3CA. Mutations to PIK3CA and PTEN loss account for 10 – 30% and up to
35% of all mCRC cases, respectively (53). The predictive and prognostic value of these two
downstream targets are difficult to assess due small patient sizes in individual trials and
therefore pooled analyses from several randomized trials have been performed (57, 58). In
patients with mCRC that receive anti-EGFR antibody treatment, the presence of wild type KRAS
and either PIK3CA mutations or PTEN loss have been reported to predict for no drug response
and poorer OS. All these mutations promote the activation of the downstream signalling
pathways; and therefore, blockage of EGFR signalling with these two antibodies to treat mCRC
becomes futile. However, currently only the determination of the KRAS and NRAS mutation
status is required for all mCRC patients prior to selection for EGFR-targeted chemotherapy.
Administration of cetuximab and panitumumab are only restricted to patients with wild-type
RAS tumours.
These studies highlight the paucity of biomarkers for most drugs used in mCRC. It is critical that new biomarkers are identified to aid in patient drug/dose selection so as to improve chemotherapy response and survival.

1.4 Cancer-associated inflammation

There is a well-established link that exists between chronic inflammation and cancer formation. At least 15% of malignancies worldwide are accounted for by chronic inflammation (59). This is apparent in CRC as patients with inflammatory bowel diseases, such as Crohn’s Disease and ulcerative colitis, are six-times more likely to develop CRC in comparison to healthy individuals, and account for 1 – 2 % of all cases of CRC (60). The risk of developing inflammatory bowel disease-associate CRC is increased with extended durations of inflammatory colitis leading to colorectal dysplasia.

Within the local tumour microenvironment of small, slowly growing tumours, the host immune response is predominantly driven by T lymphocytes and mediates an anti-tumour response (61). However, in a large, more rapidly growing and metastatic tumour, the inflammatory response within the tumour microenvironment is mediated by myeloid cells, such as tumour-associated macrophages/neutrophils and myeloid-derived suppressor cells (62). Most of the immune and stromal cells within this tumour microenvironment have dual functions in promoting and inhibiting carcinogenesis, and it is the imbalance of these roles that dictates the fate of the tumour (63). As the tumour becomes more advanced and aggressive, a communication network mediated by a complex plethora of soluble inflammatory mediators, such as interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF), between the tumour cells and immune cells is established. This communication within the local immune response stimulates the production and secretion of soluble mediators such as more cytokines, proliferative and angiogenic growth factors, chemokines, and matrix-degrading enzymes into the systemic circulation to further initiate extracellular matrix (ECM) remodelling and promote
immunosuppression of the host response, creating an environment that is favourable for sustained tumour growth, invasion and metastasis (Figure 2) (64-68).

**LOCAL IMMUNE RESPONSE**
Communication within the tumour microenvironment is mediated by secreted factors from proliferating tumour cells (+), infiltrating immune cells (-), and stromal cells (*) to regulate tumour progression.

**SYSTEMIC IMMUNE RESPONSE**
Soluble mediators within the tumour microenvironment are secreted into the systemic circulation to mediate the communication at distant sites and further promote carcinogenesis.

**Figure 2.** Relationship between local and systemic immune response during carcinogenesis. Communication between the local and systemic immune response is mediated by soluble mediators produced by the malignant, immune and stroma cells within the tumour microenvironment for the recruitment of immune cells, promotion of tumour growth and ECM remodelling. Image modified from Diakos et al. Lancet Oncology. 2014; 15(11): e493 – 503 (68).

Many of the inflammatory proteins produced by ECM remodelling in the tumour are secreted and detectable in the systemic circulation. Thus, in mCRC, the immune response mediates ECM remodelling in the tumour to enhance tumour growth and metastasis. Markers of these processes may be potential, new predictive biomarkers of the status to the tumour microenvironment.
1.5 The extracellular matrix

1.5.1 Properties, function and deregulation of ECM

The classical view of the role of the ECM is that it forms and maintains a supportive structure for normal tissue morphology; it can influence cell shape, development, proliferation, migration, survival and function (69). The functionality of the ECM is dependent on the highly dynamic structure and organisation of the ECM components. Components of the ECM include proteins such as hyaluronan, glycoproteins, proteoglycans, polysaccharides, elastin, collagen, laminin, fibulin-1 and fibronectin; integrins which are cell-surface molecules responsible for the communication and attachment between the cellular and molecular components in the ECM; and proteolytic enzymes (matrix metalloproteases (MMPs) and tissue inhibitors of MMPs (TIMPs)). Regulation and remodelling of the ECM is mainly controlled by a tight-network of proteolytic enzymes and integrins, and is mediated by two means: 1) ECM production, deposition, and degradation and 2) interactions between cells and ECM components (70, 71).

In cancer, the expression of many of these ECM enzymes and components, and various ECM tightly-regulated feedback mechanisms are deregulated. As a result, the structural integrity and framework of the ECM is compromised with altered production, deposition and turnover of ECM proteins providing proliferative and survival signals for malignant cells; and activating and stimulating integrin signalling pathways that allow for the formation of favourable tumour niches which further promotes ECM synthesis, cell growth and invasion (72, 73). MMPs can also cleave ECM-bound growth factors, such as insulin-like growth factor binding proteins and transforming growth factor-β, to allow them to become bioavailable and bind to their corresponding receptors to stimulate cell growth, migration or angiogenesis (74). Tumour invasion and angiogenesis can be further promoted directly by tumour cells secreting a variety of active MMPs into the tumour microenvironment and indirectly via inducing host cells to produce bioactive components that can assist in the degradation the ECM (72). ECM remodelling is indicative of highly invasive and metastatic tumours, resulting in poorer patient
survival (72). **Figure 3** is a schematic of the process that takes place during ECM remodelling from normal physiology to the promotion of carcinogenesis; increases production and turnover of ECM components, and cleavage of ECM-bound growth factors, activate integrins and corresponding receptors to stimulate signalling pathways to further promote cell growth, angiogenesis, and metastasis.
Figure 3. ECM remodelling promotes carcinogenesis.
Normal ECM dynamics maintain tissue integrity and homeostasis of epithelial, immune and residential fibroblasts. During carcinogenesis, the lost of ECM organisation and integrity becomes apparent as increased ECM components are degraded, produced and deposited, normal epithelial cells undergo transformation changes to become malignant, and immune cell and fibroblasts are re-educated to promote a favourable tumour microenvironment for the malignant cells to migrate, invade and metastases. Image adapted from Frantz et al. Journal of Cell Science. 2010; 123(24): 4195 – 4200 (75).
1.5.2 Link between systemic inflammation and ECM remodelling

An increasing number of studies support the importance of ECM remodelling in the tumour microenvironment, not only for deregulating cellular and structural functions, but also for altering many aspects of the immune response including infiltration, differentiation and activation of immune cells (72). Fragments of degraded ECM components, matrikines, directly act as inflammatory stimuli and chemo-attractants for immune cells (76). Therefore, abnormal ECM dynamics can either disrupt the immune response by preventing the normal activation and differentiation of immune cells, or recruit immune cells which will further promote a pro-tumour immunity within the tumour microenvironment.

Upon the initiation of an immune response within the tumour microenvironment, recruitment of innate immune cells such as macrophages and neutrophils require the ability to migrate across the endothelial cell barrier and into the tumour site. Proteolytic enzymes, MMPs, have been found to be involved in the recruitment process of immune cells, and are secreted by the immune cells to remodel the ECM (77). This process involves promoting epithelial-mesenchymal transformation by degrading E-cadherin, the formation of cell-cell and cell-ECM interactions and complex cell signalling pathways to induce cell motility (78, 79). The expression and activity of MMPs is tightly regulated by chemically reactive molecules, growth factors, cytokines, oncogenes, endogenous enzymatic inhibitors, TIMPs and ECM proteins (77, 80). Increased expression of MMPs is stimulated by pro-inflammatory cytokines such as TNF, IL-1, and IL-6, whilst anti-inflammatory cytokines such as IL-10 enhance TIMP-1 production to decrease MMPs expression; activation of MMPs can be also influenced by reactive oxygen species which are produced in large quantities by activated neutrophils and macrophages (77).

MMPs have been found to regulate systemic inflammation by altering the activity of cytokines and chemokines within the tumour microenvironment to promote carcinogenesis. Pro-inflammatory cytokine, TNF, is converted from a membrane-bound precursor into the soluble
active form by proteolytic cleavage with several MMPs including MMP-1, -2, -3, -9, -12, -14, -15, and -17 (81). Chemokine gradients are generated by MMPs modulating the bioavailability and activity of chemokines; a study conducted by Li et al in mice found that MMP-7 indirectly regulates the bioactivity of chemokine (CXC motif) ligand 1, CXCL1, by cleaving syndecan-1 from cell surfaces which releases syndecan-1 and CXCL1, generating a concentration gradient of CXCL1 to increase neutrophil recruitment (82).

1.5.3 Implications of ECM remodelling regarding chemoresistance

Implications of ECM remodelling in the tumour microenvironment, aside from the growth, invasion and metastasis advantage to tumour cells, involve the promotion of chemoresistance in malignant cells. ECM composition and organisation within the tumour microenvironment influences drug concentration and sensitivity of chemotherapy agents on tumour cells by functioning as a barrier to cytotoxic drug agents (83). As a result, tumour response to chemotherapy is reduced and the successful use of chemotherapy is compromised. This phenomenon is easily accounted for by comparing the effectiveness of cytotoxic drug agents on 2D and 3D cell culture models in vitro. CRC cell lines grown as 3D cultures (cancers with “complex” ECM structures) were found to be more resistant and insensitive to targeted EGFR inhibition in comparison to the same cell line growing in a monolayer (84). The mechanism for inducing chemoresistance in tumour cells is thought to be due to increased expression of cell adhesion molecules, cadherins and integrins, resulting in enhanced cell-to-cell and cell-to-ECM interactions within the tumour microenvironment (85). Chemoresistance is a leading cause for treatment failure in cancer patients. By understanding how ECM remodelling can influence drug response via the promotion of chemoresistance, enhanced response rates will ensure patients have improvements to their quality of life and survival outcomes.
1.6 Identification of novel biomarkers of chemotherapy response and clinical outcomes

Extensive research has been conducted in the identification of predictive biomarkers for chemotherapy response. This has primarily focused on candidates involved in the metabolism and transportation of the drug agents, as well as the VEGF and EGFR signalling pathways (46). However, there is less attention in investigating biomarkers that are associated with the tumour microenvironment and host immune response, as both these components are vital aspects of carcinogenesis (63, 86). Inflammatory markers and mediators consist of circulating immune cells, cytokines, inflammatory proteins and acute phase proteins that are detectable and measurable in the circulation. Thus, inflammatory biomarkers may provide an indication of the development and progression of cancer as well as the efficacy of treatment in cancer patients.

1.6.1 Inflammatory mediators

Cytokines, chemokines and other soluble mediators secreted by malignant or host cells to orchestrate the cellular interactions within the tumour microenvironment allow for the successful progression of malignant cells during carcinogenesis. Elevated levels of pro-inflammatory cytokines such as IL-6, IL-8, IL-10 and IL-17, are easily detected in pg/mL concentration by commercially available ELISAs. They have been found to be independently associated with poor prognosis in CRC patients, in terms of disease-free survival and OS (87-90). Chemoattractant cytokines, or chemokines, serve many roles in carcinogenesis from the recruitment and activation of neutrophils, inducing angiogenesis via CXCL1 and CXCL8, and promoting tumour growth with CXCL1 and its receptor, chemokine (CXC motif) receptor 2, CXCR2 (91). Many studies have reported that elevated chemokines have predictive and prognostic ability. Elevated expression of CXCL1 is associated with more invasive and metastatic CRCs and poorer survival outcomes in CRC patients (91-93). Secretion of these
inflammatory cytokines and chemokines also promotes angiogenesis by mediating the recruitment and activation of endothelial cells and the production of VEGF (64). Increased VEGF expression levels in CRC patients are associated with treatment failure and poorer survival (94, 95).

1.6.2 Immune cells in the local tumour microenvironment and systemic circulation

Many studies have investigated the prognostic value of immune infiltrate within the tumour microenvironment of cancer patients by evaluating the density, location and phenotype of immune infiltrate in the tumour. The Galon Immunoscore is based on scoring two subtypes of T cells (cytotoxic and regulatory T cells which are anti- and pro-tumour, respectively) according to cell surface markers CD3, CD8 and CD45RO in the tumour core and invasive margin (the interface where the host stroma meets the invading edge of a tumour) of resected tumours (96). The Galon Immunoscore classifies cancers to aid in predicting prognosis in cancer patients; an Immunoscore of 0 indicates low densities of both cell types in both regions, while an Immunoscore of 4 indicates high cell densities are found in both regions (96). Another scoring method of immune infiltrate is the Klintrup-Makinen (KM) grade, which utilises the histopathological H&E assessment of the density and inflammatory cell reaction of lymphoid, granulocytes and macrophages within the tumour core and invasive margin (97). A score of 0 indicates an absence of immune cells and a score of 3 denotes a prominent presence of immune cells and destruction of cancer cells (97). In CRC, high Galon Immunoscore and KM grades indicate high immune cell density and correlates with better prognosis (98, 99).

Recently, a new tumour microenvironment-based prognostic score has been developed which accounts for the components of the tumour microenvironment such as tumour immune cell infiltrate and stroma. The Glasgow Microenvironment Score (GMS) utilises the combination of the KM grade and the tumour stroma percentage to stratify patients with stage I – III CRC and survival outcomes (100). Three prognostic groups were classified: a GMS score of 0 indicates a
strong KM grade (a high presence of immune cells), a GMS score of 1 indicates weak KM grade/low tumour stroma percentage, and a GMS score of 2 indicates weak KM grade/high tumour stroma percentage (a low presence of immune cells and a high proportion of tumour cells); the 5-year survival rate of these groups are 89%, 75% and 51%, respectively (100).

However, all of these tumour immune infiltrate histology or immunohistochemical scores heavily rely on having access to tumour specimens. Patients with Stage I – III CRC predominantly have surgery to prevent future recurrences. However, in Stage IV mCRC, very few patients have surgical removal of the tumour, unless there is a symptomatic reason for removal, due to the fact that the outcome is relatively futile. Therefore, these scores may have limited use in mCRC patient prediction of treatment response or prognosis. Measurements of circulating immune cells and soluble mediators in the circulation therefore may be of greater benefit.

There is substantial communication between the local tumour microenvironment and systemic circulation (66, 68). The presence of cytokines, chemokines and growth factors produce inflammatory gradients to and from the tumour to promote immune influx to the tumour and efflux signals for the spread of tumours to distant sites. Measurement of the immune cells within the circulation may provide insights into the status of tumours during carcinogenesis and play a vital role in determining the clinical outcome of cancer patients (101). Neutrophils account for the highest abundance of immune cells in the circulation and clinical measurements of circulating neutrophil and lymphocyte counts have been shown to predict clinical outcomes and response to chemotherapy in cancer patients (68). The neutrophil-lymphocyte ratio (NLR) is a surrogate marker of systemic response and has repeatedly been reported to have prognostic value in cancer patients (102, 103). In CRC, patients with NLR > 5 were found to have poorer median OS (8 months shorter than patients with NLR ≤ 5) and poor response to chemotherapy (104). Furthermore, normalisation of NLR to less than 5 after one
cycle of FOLFOX improved survival in comparison to patients who remained to have NLR > 5 (104). High levels of circulating monocytes and myeloid-derived suppressor cells have been reported to be associated with increasing tumour stage and poor prognosis in CRC patients (105-107).

**1.6.3 Acute phase proteins**

Acute phase proteins (albumin, amyloid A and C-reactive protein (CRP)) are non-specific inflammatory proteins produced by the liver and secreted into the systemic circulation in response to any form of inflammation such as rheumatoid arthritis, cardiovascular disease, allergic airway diseases and cancer, which generally have increased levels of acute phase proteins (108-111). Acute phase proteins can also act as markers of systemic inflammatory response, are clinically measured in the μg – mg/mL range and provide an indication of inflammatory disease status and cancer (111).

Elevated levels of CRP are found in cancer patients in comparison to healthy individuals and are associated with poor prognosis (112). The combination of clinically measured CRP and albumin concentration result in the Glasgow Prognostic Score (GPS) which has also been consistently reported to have prognostic value in cancer patients (113). A GPS of 0 indicates CRP levels < 10mg/L and albumin > 35g/L, a GPS of 1 indicates either CRP > 10mg/L or albumin < 35g/L, and a GPS of 2 indicates CRP > 10mg/L and albumin < 35g/L. Generally, patients with GPS > 1 were found to have poor OS. Use of GPS has also been shown to predict response in metastatic lung cancer patients treated with platinum-based chemotherapy; where patients with GPS > 1 had reduced chemotherapy response rates (114). However, it is still not completely understood why patients with elevated systemic inflammation have poorer clinical outcomes.
The systemic inflammatory status of a patient has been shown to influence the metabolism of drugs. Patients with elevated systemic inflammation have reduced expression and activity of cytochrome P450 3A (a principle enzyme which metabolises a variety of chemotherapy agents) (115, 116). This consequently raises concerns for the safety and efficacy of administrating chemotherapy agents in cancer patients with elevated systemic inflammation. In a recent study conducted by Laird et al., CRP levels was found to be associated with common symptoms such as nausea/vomiting, fatigue, pain, anorexia, loss of appetite, and cognitive, physical, social and emotional dysfunction (101). The most well-established and extreme clinical manifestation as a result of systemic inflammation is the cancer cachexia syndrome, which involves the significant loss of skeletal muscle and adipose tissue independent of nutritional intake, widespread hyper-metabolic changes, and poor quality of life, physical function and survival (117, 118). Therefore, the presence of an elevated systemic immune response would be useful in predicting which patients would tolerate and respond to chemotherapy, as well as those who are more susceptible to being associated with poorer clinical outcomes.

1.6.4 ECM remodelling biomarkers

The role of the ECM in carcinogenesis has been receiving increasing attention with its integral involvement in initiating cancer growth, invasion, migration and angiogenesis within the tumour microenvironment. Tumour budding reflects the detachment of malignant cells from the tumour core at the invasive margin into isolated single cells or clusters of cells; and is assumed to characterise the initial phase of metastasis and involvement of ECM degradation. Tumour stromal percentage reflects the proportion of malignant cells to the surrounding stroma and provides an indication of ECM remodelling and deposition (119). Both tumour budding and tumour stromal percentage characteristics are assessed and scored with routine histopathological H&E staining. High tumour budding and tumour stroma percentage provide an indication of vigorous remodelling of the ECM and are associated with increasing tumour grade and poorer survival (119-121). Increased expression of proteins involved in migration,
angiogenesis and ECM remodelling such as MMPs, CD44 (cell surface molecule involved in cell adhesion and migration), tubulin, laminins, urokinase plasminogen activator receptor, VEGF, and fibronectin, has been associated with high-grade tumour budding (122, 123).

Numerous ECM components have been found to be secreted in the circulation with varying concentrations ranging from as low as pg/mL to mg/L, and may potentially be useful as novel biomarkers of patient outcomes. However, the utility of circulating ECM proteins as a biomarker in mCRC has not been extensively investigated. There are a small selection of secreted ECM proteins which serve as possible biomarkers candidates in predicting chemotherapy response and prognosis in CRC such as vitronectin, fibrinogen, TIMP-1, MMPs, laminin and periostin (124-130) which are summarised in Table 1. None of these ECM protein candidates are in clinical use as the sample size of these studies is too small and needs to be further validated in a larger cohort. Ideally, biomarkers approved for clinical use must be readily accessible for analysis in a stable and reproducible detection assay with optimal sensitivity and specificity, and represent the tumour burden during the course of the carcinogenic process before submission for FDA approval (131).

An alternative ECM protein that is detectable in blood at high concentrations (μg/mL) is the glycoprotein, fibulin-1 (132, 133). Fibulin-1 is also found to be present in the ECM and interacts with many other ECM proteins to regulate numerous aspects of carcinogenesis. Therefore fibulin-1 could serve as a novel prognostic biomarker candidate.
### Table 1. Secreted ECM protein biomarker candidates in colorectal cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Protein</th>
<th>Type of marker</th>
<th>Summary</th>
</tr>
</thead>
</table>
| Randall (124)          | Vitronectin and    | Predictive     |  ‣ Plasma samples from mCRC patient treated with FOLFOX (n = 29) were collected prior to treatment  
                             | Fibronectin        |                | ‣ Reduced levels of vitronectin and elevated levels of fibronectin were found in mCRC patients whom were not responsive to FOLFOX chemotherapy |
| Sun *et al.* (125)     | Fibrinogen         | Prognostic     |  ‣ Blood samples were obtained prior to surgery from 255 colon cancer patients (without metastasis) receiving surgery as their primary form of treatment  
                             |                    |                | ‣ Poor disease-free survival and OS was associated in patients with high preoperative fibrinogen levels  
                             |                    |                | ‣ Elevated fibrinogen levels were associated with increasing tumour size, TNM stage, high levels of carcinoembryonic antigen, modified GPS grade, white blood cell counts, NLR and platelet-lymphocyte ratio (PLR). |
| Aldulaymi *et al.* (126)| TIMP-1             | Prognostic     |  ‣ Blood sample collected from mCRC prior to FOLFIRI treatment (n = 88)  
                             |                    |                | ‣ High levels of plasma TIMP-1 were associated with poor objective response to chemotherapy, time-to-progression and OS |
| Saito & Kameoka (127)  | Laminin            | Prognostic     |  ‣ Preoperative serum laminin levels were measured in 205 CRC patients (n = 52 with hepatic metastases and n = 153 without hepatic metastases)  
                             |                    |                | ‣ Mean serum laminin was higher in CRC patients with metastasis  
                             |                    |                | ‣ Survival rates were lower in patients with elevated laminin levels |
| Ben *et al.* (128)     | Periostin          | Diagnostic and prognostic |  ‣ Blood was collected from 67 CRC previously untreated patients (45% were advanced disease) prior to surgery, 57 patients with benign colorectal adenomas or polyps  
                             |                    |                | ‣ Serum periostin levels were significantly elevated in CRC patients and in patients with metastasis  
                             |                    |                | ‣ CRC patients with normal levels of periostin had better survival than patients with elevated levels |
| Kostova *et al.* (129) | MMP-2, -7 and -9   | Prognostic     |  ‣ CRC patients without metastasis (n = 82) had blood collected before surgery  
                             |                    |                | ‣ Preoperative levels of MMP-2, MMP-7 and MMP-9 were positively correlated with tumour stage and poor patient outcomes (however, the study did not mention what outcomes these were) |
| Maurel *et al.* (130)  | MMP-7              | Prognostic     |  ‣ Serum MMP-7 was assessed in 216 CRC patients (n = 96 without metastasis and n = 120 advanced CRC patients)  
                             |                    |                | ‣ Elevated MMP-7 was observed in patients with advanced CRC  
                             |                    |                | ‣ In mCRC patients, elevated MMP-7 levels were associated with decreased OS |
1.7 Fibulin-1

Fibulin-1 is a secreted ECM protein and plasma glycoprotein belonging to the fibulin family and was first discovered and isolated from human placenta by Argraves et al. in 1989 (134, 135). It has three modular domains (I, II and III), typical of ECM modules as shown in Figure 4.

The amino-terminal domain I consists of three anaphylatoxin-like (AT) modules (which are approximately 40 amino acids long and consist of four to six cysteine residues) and is found to be a compact α-helix structure stabilised by three disulphide bridges; the central domain II comprises of nine epidermal growth factor (EGF)-like modules (also approximately 40 amino acids long with three disulphide bridges), with eight allowing for calcium binding (cbEGF-like modules); and the carboxyl-terminal domain III features a module shared by all members of the fibulin family (FC module) and varies in length according to alternate splicing (135, 136). Alternative messenger RNA splicing of exons in the carboxyl-terminal domain III in the gene, FBLN1, encode for four splice variants, fibulin-1-A, -B, -C and -D, with domain III eliminated completely in variant A and shortened in variant B and C (35 and 117 residues, respectively) in comparison to the complete variant D (137 residues) (137, 138). Variants 1A and 1B are...
detected in the human placenta at low levels, while variants 1C and 1D are the more predominant forms expressed in various tissues from other organs in the body (138).

1.7.1 Cellular fibulin-1 expression

Fibulin-1 is widely expressed in most human adult tissues and cultured non-malignant cells, such as fibroblasts and smooth muscle cells. The localisation of fibulin-1 is most prominent in the ECM of various organs and is associated with connective tissue matrix fibers, basement membranes in various tissues and platelets (139, 140). There are limited studies that have investigated the regulation of fibulin-1 expression during cancer development and progression. Steroidal hormones such as oestrogen and progesterone have been found to increase the expression of fibulin-1 in endometrial stromal cells, and ovarian and breast cancers (141-146). However, this observation is contrary to other cancers where promoter hypermethylation has been shown to downregulate tissue fibulin-1 expression in many tumour types, including gastric, renal, hepatocellular, oesophageal, cutaneous melanoma and bladder cancers (147-152). In advanced CRC and prostate cancer, promoter hypermethylation was not observed and an overexpression of the chemokine CXCL1 was found to transcriptionally downregulate cellular fibulin-1 expression in colonic tumours (153-155).

1.7.2 Secreted fibulin-1 expression

Like many other ECM proteins, fibulin-1 is secreted and deposited into the extracellular milieu where it interacts and binds with other components and proteins of the ECM. There are limited studies which have investigated the expression of secreted fibulin-1 in cancer. In the study conducted by Clinton et al. (156), secreted (and cellular) fibulin-1 expression in ovarian cancer cell lines were increased in the presence of oestradiol. Secreted fibulin-1 expression has also been found to be regulated by one of the MMP enzymes, MMP-13, through proteolysis (157). The expression of MMP-13 has been found to be high in advanced stages of CRC and elevated expression is associated with an invasive and metastatic phenotype as well as poorer
prognosis (158, 159). However, no studies have investigated the expression and regulation of secreted fibulin-1 in CRC.

1.7.3 Functional role in cell physiology

Extensive research has identified that fibulin-1 can interact with a diverse array of proteins (Table 2). Due to its broad distribution in tissue and ability to interact with vast number of ECM proteins, it is suggested that fibulin-1 plays significant roles in cell physiology and carcinogenesis by promoting platelet adhesion in haemostasis and thrombosis; providing structural integrity and organising matrix constituents in the ECM; and regulating angiogenesis, intercellular communications and cell growth, motility and adhesion (139, 140, 160-165).

Table 2. Protein ligands interacting with fibulin-1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Fibronectin</td>
<td>Suppresses fibronectin-regulated cell adhesion and motility; incorporation into ECM</td>
<td>(142, 161, 166)</td>
</tr>
<tr>
<td>Laminin</td>
<td>Incorporation into ECM; organisation of ECM; possibly involved in cell adhesion</td>
<td>(165, 167)</td>
</tr>
<tr>
<td>Aggrecan, Versican</td>
<td>Organise ECM composition and structure</td>
<td>(163)</td>
</tr>
<tr>
<td>Elastin</td>
<td>Provides structural integrity to elastic fibres</td>
<td>(139)</td>
</tr>
<tr>
<td>Nidogen</td>
<td>Incorporation into ECM;</td>
<td>(165, 168, 169)</td>
</tr>
<tr>
<td>Endostatin</td>
<td>Possible inhibition of angiogenesis</td>
<td>(170, 171)</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>Cell proliferation, migration and adhesion; possible initiation of angiogenesis</td>
<td>(164)</td>
</tr>
<tr>
<td>ADAMTS-1</td>
<td>Cofactor to enhance ADAMTS-1-mediated proteolysis towards aggrecan; possible inhibition of angiogenesis</td>
<td>(160)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Mediates platelet adhesion; incorporated into fibrin clots to regulate haemostasis and thrombosis</td>
<td>(140, 162)</td>
</tr>
</tbody>
</table>

1.7.4 Role in carcinogenesis

Altered expression of fibulin-1 during carcinogenesis modulates the normal biological processes to further sustain the carcinogenic process. It is possible that fibulin-1 may have opposing roles in carcinogenesis (172). In ovarian and breast cancer, fibulin-1 expression is upregulated, and it is downregulated in gastric, renal, colorectal, hepatocellular, oesophageal, cutaneous melanoma and prostate cancer (143, 144, 147, 150-153, 155, 173). Not only has the
difference in expression levels indicated the tumour promoting and suppressing roles fibulin-1 may play in various cancers, but differential expression of the alternate splice variants 1C and 1D has been reported and shown to have differing roles in carcinogenesis. This disparity of function amongst the two splice variants is more evident in oestrogen-sensitive cancers where it is thought that the fibulin-1C variant is oncogenic whilst fibulin-1D is tumour suppressive; the ratio of fibulin-1C to 1D is increased in ovarian and breast cancer cell lines in comparison to normal ovarian cells (141, 142, 174, 175). A reduction or absence of fibulin-1D expression was observed in human fibrosarcoma tumour cell lines; and overexpression of fibulin-1D, resulted in a significant reduction in tumour growth in vitro and in vivo, and invasion of basement membranes was also decreased (174).

The ability of fibulin-1 to influence cell motility and adhesion is highly dependent on the ECM interaction proteins. It was found that fibulin-1 inhibited fibronectin-regulated cell attachment and motility in human breast cancer cell lines (161). As most cancers have reduced expression of fibulin-1, the inhibitory effect of fibulin-1 on cell adhesion and motility is reduced, allowing for malignant cells to progress. Cell motility and adhesion suppression by fibulin-1 via interactions between fibulin-1 and laminin, aggrecan and versican has been suggested (163, 165, 167). This suppressive property of fibulin-1 is speculated to be also regulated by the pro-angiogenic protein, angiogenin (another protein which interacts with fibulin-1), by competitive binding with other fibulin-1 binding proteins (e.g. fibronectin, laminin, versican and aggrecan) (164). Furthermore, overexpression of fibulin-1 in renal cell adenocarcinomas has been found to reduce cell proliferation, migration, invasion, and adhesion, increase apoptosis in vitro, and suppress tumourigenesis and angiogenesis in vivo (151).

Structural integrity of the ECM is essential for the normal function of tissue; modifications to the various ECM structures are associated with invasive growth in tumours. The involvement of fibulin-1 in deregulating the ECM has been pointed out in the study by Lee et al. (160),
where fibulin-1 acts as a cofactor for ADAMTS-1. ADAMTS-1 mediates the degradation of aggrecan and versican, and inhibition of angiogenesis. By co-localising with ADAMTS-1, fibulin-1 was shown to increase the activity of aggrecan cleavage. Cleavage of this ECM protein may have implications in ECM remodelling and cellular behaviours favouring the carcinogenic process. Extensive research has also investigated the anti-angiogenic and anti-tumour role of ADAMTS-1, and has shown that ADAMTS-1 levels are reduced in cancer tissue in comparison to their corresponding normal tissue (176-178). Unfortunately, Lee et al. did not explore the effect of fibulin-1 on inhibiting angiogenesis in their study (160).

The functional role of secreted fibulin-1 in carcinogenesis has only been investigated in limited studies with the use of conditioned media (CM). Xiao et al. utilised CM from renal cancer cells transfected with fibulin-1 and found tube formation was inhibited with overexpression of secreted fibulin-1 (151). Interestingly, fibulin-1 was found to be degraded by the proteolytic enzyme, cathepsin D, to produce a small fragment called neostatin (179). This fragment was then cloned and purified to evaluate its role in angiogenesis in vitro; at 10 μg/mL of neostatin, endothelial proliferation and tube formation was inhibited (179). Both of these studies suggest that secreted fibulin-1 plays a role in promoting angiogenesis.

### 1.7.5 Fibulin-1 role in predicting treatment response and survival

The study conducted by Pupa et al. is the only study to have found fibulin-1 involved in regulating the chemotherapy response in breast cancer cells (180). Enhanced fibulin-1 expression protected the breast cancer cells from chemotherapy-induced apoptosis, while silencing fibulin-1 mRNA expression or inhibiting the effects of fibulin-1 with monoclonal antibodies against fibulin-1 resulted in an increased sensitivity to doxorubicin and enhanced apoptosis (180). These results are difficult to extend to other cancer types due to alternate regulation of fibulin-1 in the majority of tumours. The prognostic role of fibulin-1 expression has been investigated in hepatocellular, cutaneous melanoma, and bladder cancer and a
reduction of fibulin-1 cellular expression in tumour tissue sections is associated with poorer survival (147-149).

Currently, the value of fibulin-1 as a marker of chemotherapy response and clinical outcomes in mCRC is still unknown. There is also little information regarding the relationships between tumour and secreted fibulin-1 expression and its functional role in CRC progression.

1.8 Thesis aims and scope

The scope of this thesis was to examine the expression and evaluate the prognostic value of fibulin-1 in its various forms in patients with mCRC. Therefore, in this thesis, I investigated the value of fibulin-1 in predicting clinical outcomes in patients with mCRC, which may offer a novel drug target for personalising treatment options and improve clinical outcomes. The expression, regulation and function of fibulin-1 in CRC were also investigated to provide an understanding of fibulin-1 in CRC carcinogenesis.

I hypothesize that a reduction in fibulin-1 expression in mCRC patients promotes ECM remodelling and tumour growth, dictating the fate of malignant cells to become an aggressive phenotype and resulting in poorer clinical outcomes in these patients.

The aims of this thesis were:

1. To confirm a reduction in fibulin-1 expression in tumour tissue of mCRC patients.
2. To investigate the expression of fibulin-1 in the plasma of mCRC patients prior to chemotherapy.
3. To elucidate the relationship between fibulin-1 expression in the tumour and plasma.
4. To evaluate the expression of fibulin-1 as a marker of chemotherapy response and clinical outcomes in mCRC patients.
5. To determine the regulatory role of the systemic inflammation on plasma fibulin-1 expression.

6. To examine the expression of all three forms (secreted, cellular and deposited) of fibulin-1, CXCL1, CXCR2 and MMP-13 in human colonic fibroblast and CRC cell lines.

7. To examine the role of CXCL1 and MMP-13 in regulating fibulin-1 expression in vitro.

8. To investigate the functional role of secreted fibulin-1 on migration and angiogenesis in CRC carcinogenesis.

9. To determine whether fibulin-1 expression and function are altered with systemic inflammation.

Specific projects undertaken to achieve these aims were:

1. Immunohistochemistry was conducted on tumour and adjacent lymph nodes tissue sections to examine tumoural fibulin-1 expression, and selective reaction monitoring mass spectrometry and Western blotting were conducted to examine and validate the expression of plasma fibulin-1 in mCRC patients (n = 32). The prognostic value of fibulin-1 expression in the tumours and plasma of mCRC patients was then evaluated.

2. Pro-inflammatory cytokine expression levels of IL-6, IL-8 and CXCL1 were measured with sandwich ELISAs. Expression of these cytokines was correlated with the expression of plasma fibulin-1.

3. Western blotting was conducted to examine cellular and secreted fibulin-1 levels and ELISAs were developed to examine the levels of fibulin-1 deposited into the ECM by colonic fibroblast and CRC cell lines.

4. Sandwich ELISAs were used to examine the cellular and secreted levels of CXCL1 in colonic fibroblast and CRC cell lines.

5. Quantitative real-time PCR was used to examine the relative mRNA levels of fibulin-1, CXCL1, CXCR2 and MMP-13.
Chapter 1 Introduction

6. Wound scratch and HUVEC tube formation functional assays were conducted on two high and two low-fibulin-1 secreting CRC cell lines to determine the role fibulin-1 plays in migration and angiogenesis, respectively.

7. Treating the cells with IL-6 in the previous *in vitro* experiments allowed us to determine whether this cytokine alters the expression and functional role of fibulin-1.
Chapter 2
Investigation of plasma fibulin-1 as a prognostic marker in mCRC patients
2.1 Introduction

Carcinogenesis is driven by a multitude of factors with an increasing recognition of the critical role of inflammation on numerous processes in aiding cancer progression (86). Studies have reported the use of markers of systemic inflammatory response such as CRP, albumin, GPS, and NLR to predict patient clinical outcomes and response to chemotherapy. Patients with elevated markers of systemic inflammation were found to have poor chemotherapy response and reduced OS (104, 113). It is still not completely understood why patients with elevated systemic inflammation have poor clinical outcomes.

There is increasing evidence which supports the relationship between local immune response within the tumour microenvironment and systemic inflammation (68). The crosstalk between these two systems is mediated by a complex plethora of soluble mediators produced by the tumour microenvironment and secreted into the systemic circulation such as cytokines, chemokines, growth factors and matrix-degrading enzymes and a host of immune and stromal cells. Inflammatory mediators, such as IL-6, initiate the systemic inflammatory response and ECM remodelling to orchestrate tumour growth, progression, invasion, angiogenesis and metastasis and educate recruited immune cells for immune evasion within the tumour microenvironment (64-67, 77). Thus, understanding the relationships of soluble mediators released during advanced stages of carcinogenesis may potentially lead to greater knowledge of the fate of a malignant cell and assist in predicting the clinical outcomes of patients with mCRC.

Numerous studies support the importance of the ECM in the tumour microenvironment, not only for regulating cellular and structural functions directly, but acting as inflammatory stimuli and chemo-attractants for immune cells via degraded fragments of ECM proteins (72, 76). In cancer, deregulation of ECM dynamics is a vicious cycle controlled by a tight-network of protein-protein interactions, proteolytic enzymes such as MMPs and their endogenous
inhibitors, TIMPs, and cell surface receptors called integrins (71, 74). ECM remodelling involves two actions: 1) synthesis and deposition of components of the ECM and 2) degradation of the ECM. Increased activity in either action is indicative of highly invasive tumours and poorer patient survival (72). There is also evidence that highlights the role of ECM organisation and composition within the tumour microenvironment in influencing drug concentration and sensitivity of chemotherapy agents on tumour cells via the interaction of cell adhesion molecules and integrins with components of the ECM (85, 181). Despite the role the ECM plays in carcinogenesis and chemotherapy resistance, there are no ECM proteins that are used clinically to predict prognosis and chemotherapy response in patients with CRC.

Fibulin-1 is a critical ECM component located within the stromal compartment of tissues and secreted as a plasma glycoprotein (135). Due to its ability to interact with various ECM components, such as fibronectin, elastic fibers, angiogenin and laminin-1, fibulin-1 has a range of functional roles that influence cellular communication and behaviour and regulate ECM structural dynamics within the tumour microenvironment. Changes in the cellular expression of fibulin-1 in tumour cells has been shown to increase apoptosis and inhibit cell proliferation, adhesion, migration, invasion and angiogenesis (142, 151, 154, 161, 179).

In CRC, a reduction of tumour fibulin-1 expression has been observed in comparison to adjacent normal mucosal tissue in patients (153). Fibulin-1 expression was significantly reduced in more advanced stages of CRC and associated with increased tumour cell proliferation (153). Cellular expression of fibulin-1 in CRC tumour cells was also found to be regulated by the inflammatory chemokine, CXCL1 (153). Repression of CXCL1 using siRNA increased the expression of fibulin-1 and inhibited the growth of CRC cell line HCT-15 in vitro. Based on these finding, it may be hypothesized that in CRC patients with inflammation, fibulin-1 is reduced leading to enhanced tumour proliferation and poorer clinical outcomes. Other
studies investigating hepatocellular, cutaneous melanoma and bladder cancer have shown that reduced fibulin-1 expression is associated with poorer survival (147-149).

Whilst the use of immunohistochemistry (IHC) has been the recommended standard method for examining clinical pathological biological specimens, they only provide a small glimpse into the local tumour microenvironment at one static time point, i.e. at diagnosis, and little information about the temporal changes or secreted versions involved during carcinogenesis. Plasma is easily accessible, can provide a snap shot of a disease state in the body at any given point in time and accessed serially for longitudinal monitoring of proteins (182, 183). Interestingly, fibulin-1 is one of the few ECM proteins found to be secreted at relatively high levels within the blood (132, 133); although, very little is known about the origin and clinical significance of fibulin-1 in the circulation. Increased fibulin-1 levels in the blood have been found in patients with respiratory diseases such as asthma and idiopathic pulmonary fibrosis, chronic kidney disease and type 2 diabetes (132, 133, 184-186). Recently, plasma fibulin-1 has been implicated in dysfunction of smooth muscle in asthma, and identified as a potential biomarker of renal impairment and arterial ECM remodelling in patients with type 2 diabetes related to arterial stiffness (132, 133, 185, 186). An increase in plasma fibulin-1 in diabetes patients was also predictive of higher overall mortality and cardiovascular mortality (132). These studies provide evidence of the role of fibulin-1 in ECM remodelling and as a prognostic marker.

There are limited studies which have investigated the expression and biological function of secreted fibulin-1 in carcinogenesis. Secreted fibulin-1 expression has only been investigated in one study which found secreted fibulin-1 expression was increased in ovarian cancer cells in the presence of oestradiol (156). Overexpression of fibulin-1 in CM of ACHN renal cancer cells was found to reduce tube formation in vitro, only to be restored by pre-treatment with anti-fibulin-1 antibody (151).
The prognostic value of plasma fibulin-1 in predicting chemotherapy response and clinical outcomes in CRC patients is still not known. Therefore, the development of a highly sensitive plasma-based assay is required to allow for the detection of fibulin-1 and determine its prognostic application in the clinic.
2.2 Aims

The aims of this chapter were:

1. To confirm the reduced tumour expression of fibulin-1 in mCRC patients.

2. To investigate the relationship between the expression of fibulin-1 in the tumour and plasma of mCRC patients.

3. To investigate the relationships between fibulin-1 and clinical outcomes in mCRC patients.

4. To determine the role of systemic inflammation in the regulation of plasma fibulin-1 expression in mCRC patients.
2.3 Method

2.3.1 CRC patient recruitment, characteristics and clinical follow-up

Patients with advanced CRC (Australian Clinicopathological Stage C & D) undergoing fluropyrimide-based chemotherapy were prospectively recruited as part of a Biomarkers in CRC study (Sydney Southwest Area Health Service research ethics committee approval #CH62/6/2006-132), from Concord Repatriation General Hospital and Royal Prince Alfred Hospital, Sydney. The study was conducted according to guidelines of the Declaration of Helsinki, and informed, written consent was obtained from all participants. Recruited patients primarily received FOLFOX as their chemotherapy regimen; which consisted of 2-week cycles of oxaliplatin (85 mg/m²) intravenously administered over 2 hours concurrently with leucovorin (400 mg/m²) and followed by 5-FU bolus (400 mg/m²) and a 46-hour continuous 5-FU infusion (2400 mg/m²). Other treatment options patients were provided included 5-FU de Gramont; which follows the same regime as FOLFOX without the addition of oxaliplatin, FOLFIRI; which replaces oxaliplatin in the FOLFOX regimen with irinotecan (180 mg/m²) intravenously administered over 30 – 90 minutes, and FOLFOX with either intravenous infusion of panitumumab (6 mg/kg) over 1 hour or bevacizumab (5 mg/kg) over 30 – 90 minutes on day 1.

Patient clinical response to chemotherapy was assessed and measured 6 weeks after treatment by the treating clinician and an independent radiologist, and graded accordingly to RECIST criteria using CT scans (187). Under these guidelines, in comparison to baseline measurements, the response criteria by definition are:

- Complete response: all lesions have disappeared
- Partial response: lesions have decreased in size by at least 30%
- Stable disease: no sufficient changes to lesion size
- Progressive disease: lesions have increased in size by at least a 20%
To examine the relationships between plasma fibulin-1 expression and clinical outcomes, the 32 patients were categorised into three response groups: responsive (partial and complete) (n = 19), stable disease (n = 7) and progressive disease (n = 6). Dates of progression and death were followed up routinely for 3 years by treating clinicians.

2.3.2 Immunohistochemistry

Tissue samples of human colorectal cancer and corresponding sites containing cancers within lymph node metastasis were collected and provided by the Department of Anatomical Pathology, Concord Repatriation General Hospital, Sydney. Tumour sections from 19 mCRC patients were available for examining fibulin-1 expression in tumour tissue; and 12 of these patients had sections available for examination of cancerous tissue in adjacent lymph node metastasis.

Two 4 µm formalin-fixed, paraffin-embedded sections were used to examine fibulin-1 expression with IHC staining. Tissue sections were deparaffinised in a series of xylene and alcohol washes before being hydrated with deionised water. Briefly, this involved two 10 minutes washes with xylene, two 2 minutes with 100% ethanol, 2 minutes with 95%, 70% and 50% ethanol, and 2 minutes in deionised water. Sections were blocked with non-immune blocking solution (Dako) for 1 hour at 37°C in a humidified chamber before incubating with mouse anti-human fibulin-1 antibody (clone B-5, 0.05 µg/mL, Santa Cruz) in antibody diluent (Dako) for 2 hours at 37°C in a humidified chamber; followed by a series of five 3 minutes washes with Tris-buffered saline (TBS)-Tween-20 buffer. Endogenous peroxidase activity was inhibited with peroxidase-blocking solution (Dako) for 15 minutes at room temperature, washed again three times and then incubated with anti-mouse EnVision™+ System-HRP secondary antibody (Dako) for 45 minutes at 37°C in a humidified chamber. Slides were washed three times and then incubated with 3,3′-diaminobenzidine (DAB) + substrate-chromogen (Dako) for up to 15 minutes at room temperature until a brown-coloured stain was
detected. A series of five 2 minutes washes in deionised water were conducted before H&E staining. Briefly, Meyer’s haematoxylin (Sigma) was used to stain the slides for 7 minutes at room temperature, and then slides were rinsed with tap water for 2 minutes and counterstained with eosin Y for 1 minute at room temperatures. Slides were dehydrated with a series of quick ethanol washes (70%, 95% and two 100%) and two 2 minutes xylene washes and then cover-slipped and mounted with dibutyl phthalate xylene (DPX) mounting medium (VWR International). For each slide, a negative isotype control (mouse IgG2a monoclonal antibody (Dako)) at the same concentration of the primary antibody was included. The positive control used for this experiment was human idiopathic pulmonary fibrosis lung parenchymal tissue obtained from resected human lungs and post mortem organ donors from St Vincent’s Hospital, Sydney; which was kindly provided by Assoc Prof Janette Burgess and Mr Gavin Tjin (Woolcock Institute of Medical Research, Sydney). Five randomly-selected images from the superficial tumour tissue (100X magnification) were obtained using a Nikon Eclipse TS100 inverted light microscope attached to a Digital Sight DS-L3 camera (Nikon). Staining intensity was assessed against the isotype control for each tissue sample and categorised as negative for no and weak staining, or positive for moderate and strong staining.

2.3.3 CRC patient plasma collection

All patients provided voluntary informed consent prior to blood collection. Blood samples were collected using a 21 gauge needle and syringe into BD Vacutainer® K2EDTA anti-coagulant tubes (Lavender closure, BD) prior to chemotherapy and centrifuged within 30 minutes of collection at 2500 g for 20 minutes at room temperature. Plasma was aliquoted in 0.5 mL volumes and stored at -80°C for long term storage.

2.3.4 Plasma preparation for selective reaction monitoring mass spectrometry analysis

The use of selective reaction monitoring mass spectrometry (SRM-MS) offers a more targeted approach for the detection and quantitation of proteins in a complex source of secreted
proteins in the plasma with high sensitivity, dynamic range, specificity and reproducibility in a single multiplexed assay and this technique is increasingly being used in clinical applications in a wide variety of diseases (183, 188).

### 2.3.4.1 Top-14 plasma protein depletion and protein precipitation

One of the problems with detecting low abundance proteins is the amount of high abundant proteins found in the plasma. Approximately 99% of the total protein mass in plasma accounts for the top 21 highly-abundant proteins, leaving a large proportion of lower abundance proteins in the remaining 1% being masked and undetected by the presence of these highly-abundant plasma proteins (189). Numerous studies have shown significant increases to protein detection by using various immunodepletion strategies (190-193). Therefore, by employing immunoaffinity strategies to deplete the top 14 high abundance plasma proteins, detection of lower abundance proteins using proteomics is enhanced and deregulated protein profiles are more easily identified. Plasma was depleted of the top 14 high abundance proteins with a MARS-14 depletion column (7.5 x 100 mm, Agilent) which selectively depletes plasma of human serum albumin, serotransferrin, haptoglobin, IgG, IgA, a1-antitrypsin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, complement C3, IgM, apolipoprotein AI, apolipoprotein AII and transthyretin.

Aliquots of plasma, 150 μL, were diluted four times the volume of sample with Buffer A (Tris-HCl and NaCl, pH 7.4) (1:4 sample:buffer) (Agilent), transferred to a 0.22 μm spin filter and spun at 16,000g for 1 minute at 4°C. The flow through sample was loaded and run with Buffer A onto the MARS-14 depletion column using the Agilent 1100 quaternary HPLC system and depleted with a two-step 0 – 100% Buffer B (Glycine-HCl, pH 2.5) (Agilent) gradient at a flow rate of 0.25 mL/min for 20 minutes. Highly abundant proteins were eluted with Buffer B at a flow rate of 2 mL/min for 5 minutes.
Proteins in the depleted plasma were precipitated overnight at 4°C with chilled acetone at a ratio of 1 (sample):10. The resulting precipitate was spun at 3,000g for 100 minutes at 4°C prior to acetone removal. The protein pellet was resolubilised and sonicated with 750 µL 0.25 M triethylammonium bicarbonate (TEAB) (Sigma Aldrich) to completely dissolve the pellet.

2.3.4.2 Protein quantitation with bicinchoninic acid protein assay

Total protein was determined by the Pierce Bicinchoninic acid (BCA) protein assay (Thermo Scientific) according to the manufacture’s protocol. Briefly, protein concentration in depleted plasma samples was measured using a colourimetric protein assay based on a shift in absorbance of BCA upon binding to protein. Samples were diluted (1:2) with TEAB and 25 µL of this diluted sample was added to 200 µL BCA working reagent and incubated at room temperature for 30 minutes. Samples were quantitated in duplicate using bovine serum albumin as a protein standard (0 – 2 mg/mL) and absorbance was measured with a FLUOstar OPTIMA plate reader (BMG LabTech) at an absorbance of 562 nm.

2.3.4.3 Reduction, alkylation and trypsin digestion of plasma samples

Aliquots of depleted plasma samples, 40 µg, were reduced with 5 µM tris(2-carboxyethyl)phosphine (TCEP) (Sigma Aldrich) at 60°C for 1 hour, alkylated with 10 µM methylmethanethiosulfonate (MMTS) (Sigma Aldrich) at room temperature for 10 minutes and digested with 1.6 µg sequencing grade modified trypsin (Promega) at 37°C for 16 hours. Digested samples were vacuum-dried for 1 hour in a vacuum centrifuge (SpeedyVac) and stored at -20°C until SRM-MS analysis.

2.3.5 Development and optimisation of SRM-MS method

2.3.5.1 Identifying peptides and transitions

High specificity of SRM-MS arises as a result of selecting two m/z ratios values, termed transitions, to distinguish and quantitate specific analytes of proteins of interest within a
complex sample (Figure 5). To verify and quantitate selected protein candidates in the mCRC patient cohort, SRM-MS transitions for fibulin-1, CRP and serum amyloid A1 (SAA1) were manually designed and optimised using MRMPilot V2.0 software (AB Sciex). The software allows for the building and optimisation of peptide SRM-MS methods using an *in silico* approach. For each protein, a list of possible SRM transitions was obtained by taking the protein’s amino acid sequence and theoretically digesting the sequence *in silico* with the following settings chosen and criteria:

- **Digest settings:** Oxidation (M), Carboxymethyl (O) (both Fixed mods) and Trypsin (Enzyme)
- **Peptide selection:** Sequence length 6 – 20 amino acids
- **Ignore peptides with methionine, tryptophan or cysteine residues and multiple modification sites**
- **Peptides needed to be doubly charged**
- **Q1 m/z values for the precursor ion and Q3 m/z values for the fragment ion varied between m/z 300 – 1200; Q3 values required to be at least two fragment y-ions above the precursor ion.**
Figure 5. A schematic of the principle of SRM performed in a triple quadrupole mass spectrometer for the fibulin-1 peptide, SQETGDLVGLQETDK. Following sample ionization, the precursor ion is selected according to the m/z value at the first quadrupole (Q1) before passing into the collision chamber (Q2) where the peptides undergo fragmentation, the fragment ions are then selected by the second m/z value at the third quadrupole (Q3).

Transitions obtained were evaluated by searching the peptide sequence in BLAST (NCBI) against protein database, UniProt, in Homo sapiens, to ensure that the peptide sequence was specific for the protein of interest.

2.3.5.2 Verifying transition specificity

To verify and confirm the presence of specific transitions for the proteins of interest, scanning data acquisition was performed in the data dependent acquisition mode to provide a spectrum of ions eluting at a precise time. In this mode, an enhanced MS scan was acquired (m/z 350 – 1200) with the three most intense multiply-charged ions subsequently subjected to two enhanced product ion MS/MS scan.

Transition specificity was reconfirmed once again by searching the MS/MS spectra data against all Homo sapiens entries in the SwissProt database using the MASCOT algorithm (Matrix Science). In MASCOT, peptides are ranked according to the probability-based MOWSE (Molecular Weight Search) score, an algorithm which uses mass values (either from peptides or MS/MS fragment ions) to calculate the probability that an observed mass value is a random
event to determine protein identification and access whether they are significant or not; scores of 33 or greater indicate significant identity or extensive homology. Based on the qualitative and quantitative information for a given transition from a patient plasma test sample, the collision energy (the energy required to fragment the precursor ion into fragment ions) was altered to further optimise the transitions with MRMPilot. However, if the transitions were deemed irreproducible or detectable, they were discarded and new transitions were acquired and evaluated. Optimised transitions for the protein candidates are listed in Table 3.
### Table 3. Optimised peptide transitions and biological function of proteins candidates for SRM-MS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Q1 m/z</th>
<th>Q3 m/z</th>
<th>Collision energy (V)</th>
<th>Elution time (min)</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibulin-1</td>
<td>TGYYFDGISR</td>
<td>589.78</td>
<td>694.35</td>
<td>30.95</td>
<td>41.46</td>
<td>Associates with ECM components to regulate dynamics in ECM and cellular behaviour; binds with fibrinogen to mediate platelet adhesion.</td>
</tr>
<tr>
<td></td>
<td>SQETGDLVGGLQETDK</td>
<td>896.42</td>
<td>847.42</td>
<td>49.00</td>
<td>37.18</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>ESDTSYVSLK</td>
<td>564.77</td>
<td>696.39</td>
<td>29.85</td>
<td>31.93</td>
<td>Acute phase protein which is increased during inflammation &amp; activates the complement cascade.</td>
</tr>
<tr>
<td>Serum amyloid A1</td>
<td>SFFSFLGEAFDGAR</td>
<td>775.87</td>
<td>765.35</td>
<td>41.10</td>
<td>72.13</td>
<td>Acute phase protein which is increased during inflammation to recruit inflammatory cells &amp; induces enzymes to degrade the ECM.</td>
</tr>
</tbody>
</table>
2.3.6 SRM-MS analysis of fibulin-1, CRP and SAA1 in mCRC patient plasma

Dried peptides from top-14 depleted plasma samples were resuspended in 80 μL 3% acetonitrile (ACN)/0.1% formic acid (FA) and run in triplicates on the 5500 QTRAP® hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex) coupled with a Waters nanoAcquity UPLC system. Samples were injected as a full loop injection for each SRM experiment replicate (10 μL). Peptides were separated using a nanoAcquity UPLC BEH C18 column (100 μm x 1.7 μm x 100 mm, Waters) and eluted from the column using a linear gradient from 3% ACN /0.1% FA to 65% ACN /0.1% FA over 130 minutes at a flow rate of 400 nL/min. Column eluent was directed into an uncoated PicoTip-fused silica spray tip (SilicaTip™, 360 μm OD x 20 μm ID x 10 μm diameter emitter orifice, New Objective), ionised into the 5500 QTrap and analysed in positive ion mode using a SRM scan type, with a total scan time of 2.08 seconds which included pauses.

Area under the curve (AUC) for each transition peak was integrated and quantitated for all replicates using the MultiQuant software (AB Sciex) using the MQ4 integration algorithm. The algorithm selects the target transition peak according to the expected retention time and peak profile, and automates this process by defining and applying various integration parameters for peak quantitation (Gaussian smoothing and reduction of peak splitting, retention time half window, peak width and height, noise percentage, baseline window). Each peak was manually evaluated to ensure for correct integration of the AUC. Otherwise, the peak was manually selected at the expected retention time for the transition.

For each targeted peptide, the AUC of the transition peaks were averaged from all the peptide replicates, the absolute abundance for each peptide was summed from the averaged transitions AUC values and reported as signal intensity (count per second (cps)) for each patient. SRM-MS analysis of fibulin-1 according to pro-inflammatory cytokines were categorised according to median signal intensity values.
2.3.7 Western blotting

A mouse anti-human fibulin-1 monoclonal antibody (clone B-5, Santa Cruz) has already been demonstrated to successfully detect serum fibulin-1 with Western blotting (194). This antibody epitope region corresponds to the amino acid sequence 1 – 190, which recognises the N-terminus of all four fibulin-1 splice variants. To validate and analyse the circulating fibulin-1 levels in mCRC patient plasma, Western blotting for the intact protein was performed.

Plasma samples were diluted in PBS (1:50) and then further diluted in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and Complete protease inhibitor cocktail, mini (Roche) (1:10). Each membrane was loaded with a positive (1% fetal bovine serum (FBS)), negative (100 ng fibronectin) and normalising (1:500 healthy plasma sample) control. Proteins were separated on 10% SDS polyacrylamide gels at 125V for 70 minutes, transferred to PVDF membranes at 25V for 1.5 hours and blocked with 5% skim milk/2% bovine serum albumin (BSA)/TBS/0.1% Tween-20 (w/v) blocking solution at room temperature for 1 hour. All antibodies were diluted in 2% BSA/TBS/0.1% Tween-20. Membranes were incubated with mouse anti-human fibulin-1 monoclonal antibody (clone B-5, Santa Cruz, 0.2 μg/mL for supernatant samples and 0.3 μg/mL for cell lysate samples) at 4°C overnight. Following five 3 minute TBS/0.1% Tween-20 washes, membranes were then incubated with goat anti-mouse IgG-HRP polyclonal antibody (DAKO, 0.2 μg/mL for supernatant samples and 0.3 μg/mL for cell lysate samples) at room temperature for 1 hour. After washing, blots were visualised and exposed with Immobilon Western Chemiluminescent HRP substrate (Millipore) for 3 minutes, bands were analysed using ChemiDoc™ MP Imaging system (Bio-Rad), and the amount of protein detected in each sample was determined based on the densities obtained with Image Lab Version 4.0.1 (Bio-Rad). The densities of fibulin-1 bands for each membrane were normalised according to the normalising factor of each membrane. For each membrane, the normalisation factor was calculated as the ratio of the 100kDa fibulin-1 band density detected in the normalising healthy plasma sample.
on the membrane against a representative membrane. Results were expressed as a fold change relative to the normalising healthy control.

$$\text{Normalisation of fibulin-1} = \frac{\text{fibulin-1 band density} \times \text{Normalisation factor}}{\text{Normalising control band density}}$$

2.3.8 Measurement of inflammatory cytokines CRP, IL-6, IL-8 and CXCL1

Prior to chemotherapy, routine laboratory measurement of serum CRP was conducted as a marker of inflammation and to monitor response to chemotherapy. The expression of IL-6 and CXCL1 were measured using human IL-6 and CXCL1 Quantikine® ELISA assays, respectively, and IL-8 was measured with human IL-8 Quantikine® High Sensitivity ELISA assay (R&D) according to manufacturer’s protocol. Briefly, plates had assay diluent added to each well before 100 μL of plasma (200 μL for CXCL1) and a standard curve was incubated in duplicates for 2 hours at room temperature. Plates were washed with wash buffer at least three times before 200 μL of conjugate polyclonal antibody was added and then washed at least three times. Standard curve concentration and incubation times and temperatures for each ELISA kit was summarised in Table 4.

<table>
<thead>
<tr>
<th>ELISA reagent</th>
<th>IL-6</th>
<th>IL-8</th>
<th>CXCL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard curve concentration</td>
<td>Seven point; 0 – 100 pg/mL</td>
<td>Eight point; 0 – 64 pg/mL</td>
<td>Six point; 0 – 500 pg/mL</td>
</tr>
<tr>
<td>Conjugate polyclonal antibody</td>
<td>2 hours at room temperature</td>
<td>1 hour at room temperature</td>
<td>1 hour at 4°C</td>
</tr>
</tbody>
</table>

Then 200 μL of substrate solution (1:1 colour reagent A (hydrogen peroxide) and colour reagent B (tetramethylbenzidine)) were incubated for 30 minutes at room temperature and protected from light (for IL-8, 50 μL of substrate solution was incubated for 1 hour at room temperature and an additional 50 μL of amplifier solution for 30 minutes at room temperature to enhance and initiate the colour change reaction), before the reaction was terminated with 50 μL 1 M H₂SO₄. Absorbance was read within 30 minutes at 450 nm and 570 nm using a FLUOstar Omega spectrophotometer (BMG LabTech). Raw values of IL-6, IL-8 and CXCL1 were
corrected by calculating the difference in absorbance at 540 nm from 450 nm, and concentrations were calculated using a four parametric logistic fit of the standard curve ($r^2 > 0.99$) with MyAssays (http://www.myassays.com/quantikine.assay, R&D).

### 2.3.9 Statistical analysis

Statistical analyses were performed with SPSS Version 21.0 (IBM). Patients were categorised into three response groups: responsive (partial and complete), stable disease and progressive disease according to RECIST criteria. Clinical benefit from chemotherapy was defined as responsive and stable disease, and no benefit as progressive disease. Progressive-free survival (PFS) was analysed as a categorised variable of PFS dichotomized at 1 year. OS was analysed in two ways; 1) as a continuous variable using the Kaplan-Meier method with log-rank test and 2) as a categorised variable with OS dichotomised at 3 years. Fisher’s exact test was conducted to examine the relationships between expression of tumour and plasma fibulin-1 and the association of NLR with clinical benefit to chemotherapy and clinical outcomes. Non-parametric Mann-Whitney U-tests and Spearman’s correlation (where $r$ is Spearman’s coefficient) were conducted to analyse the relationships between NLR, CRP, SAA1, fibulin-1, secreted cytokines levels and clinical outcomes, unless specified otherwise. Values were expressed as median and individual results; and significance was determined as p-value < 0.05.
Chapter 2 Investigation of plasma fibulin-1 as a prognostic marker in mCRC patients

2.4 Results

2.4.1 Patient characteristics

Thirty-two mCRC patients were included in the study. The demographics, chemotherapy response and clinical outcomes of the included patients are summarised in Table 5. These patients were predominantly male and had a median age of 60 (range 24 – 75) years. All but one patient had a good Eastern Cooperative Oncology Group (ECOG) performance status of < 1 and six patients had evidence of elevated systemic inflammation as determined by NLR > 5. Twenty-nine patients had received FOLFOX based chemotherapy; and of these patients, five had FOLFOX in combination with bevacizumab and one had FOLFOX in combination with panitumumab. Two patients were treated with FOLFIRI and one was treated with 5-FU de Gramont chemotherapy regimen. As these chemotherapy treatments have similar response rates (50 – 55%), with the exception of the 5-FU de Gramont chemotherapy regimen which has a response rate of approximately 22% (21, 24, 39, 43); the response to chemotherapy for all patients in this cohort was collated and included for analysis to simplify the investigating between fibulin-1 expression with chemotherapy response. The patient treated with 5-FU de Gramont had a partial response to their chemotherapy, and upon calculating the overall clinical response rate (complete and partial response) to chemotherapy for this cohort, if this patient was excluded from further analysis the overall response rate would be 58%, as oppose to 59% if the patient was included. Therefore, this patient was included in further analysis. Clinical benefit (responsive and stable disease) to chemotherapy was observed in 81% of patients.

The follow up period commenced at the start of chemotherapy to the censor date of April 2014, with time censored for patients lost to follow-up or who were alive at the end of the study. The median PFS of the patient cohort was 296 days (9.7 months); and the median OS for the entire cohort was 858 days (28.13 months) (range 68 – 2027 days) and in censored patients, the median OS was 1456 days (47.74 months) (109 – 2027 days). Within this follow
up period, 94% of patients had progressed with chemotherapy and 66% patients were deceased. Consistent with current literature, patients with elevated systemic inflammation (as indicated by NLR > 5) had reduced clinical benefit from chemotherapy (p = 0.06) (Figure 6A) and poorer PFS and OS (p = 0.03 and p < 0.0001, respectively) (Figure 6B & C).

Table 5. Patient characteristics and clinical outcomes in mCRC cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (63)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (37)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>60</td>
</tr>
<tr>
<td>Range</td>
<td>24 to 75</td>
</tr>
<tr>
<td>ECOG PS</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>21 (66)</td>
</tr>
<tr>
<td>1</td>
<td>10 (31)</td>
</tr>
<tr>
<td>2</td>
<td>1 (3)</td>
</tr>
<tr>
<td>NLR</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>2.9</td>
</tr>
<tr>
<td>Range</td>
<td>1.6 to 11.4</td>
</tr>
<tr>
<td>High (&gt;5)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>Low (&lt;5)</td>
<td>26 (81)</td>
</tr>
<tr>
<td>RECIST Response</td>
<td></td>
</tr>
<tr>
<td>Responsive (Complete/Partial)</td>
<td>19 (59)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>7 (22)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>6 (19)</td>
</tr>
<tr>
<td>PFS</td>
<td></td>
</tr>
<tr>
<td>Median (days)</td>
<td>296</td>
</tr>
<tr>
<td>Range (days)</td>
<td>4 to 1964</td>
</tr>
<tr>
<td>PFS &lt; 1 year</td>
<td>20 (62)</td>
</tr>
<tr>
<td>PFS &gt; 1 year</td>
<td>12 (38)</td>
</tr>
<tr>
<td>OS</td>
<td></td>
</tr>
<tr>
<td>Median (days)</td>
<td>858</td>
</tr>
<tr>
<td>Range (days)</td>
<td>68 to 2027</td>
</tr>
<tr>
<td>OS &lt; 3 years</td>
<td>21 (66)</td>
</tr>
<tr>
<td>OS &gt; 3 years</td>
<td>11 (34)</td>
</tr>
</tbody>
</table>

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; NLR = Neutrophil/Lymphocyte ratio; RECIST = Response Evaluation Criteria In Solid Tumours; PFS = Progressive free survival; OS = Overall survival
Figure 6. Analysis of clinical benefit from chemotherapy and clinical outcomes according to NLR. Patients with elevated systemic inflammation (NLR > 5, n = 6) had less clinical benefit to chemotherapy (p = 0.06) (A) and poorer PFS (B) and OS (C) compared to patients with low systemic inflammation (NLR < 5, n = 26).

2.4.2 Reduced tumour fibulin-1 expression

Tumour sections from 19 mCRC patients were available for examining fibulin-1 expression in tumour tissue; and 12 of these patients also had sections available for examination of cancerous tissue in adjacent lymph nodes. Characteristics of these patients are summarised in Table 6. The patients were predominantly male and had a median age of 60 (range 24-75) years. All patients had a good ECOG performance status of < 1 and four patients had elevated systemic inflammation as determined by NLR > 5. Of the 19 mCRC patients, 17 were treated with FOLFOX and two patients were treated with FOLFIRI. The overall clinical response rate (complete and partial response) was 53% and clinical benefit (responsive and stable disease)
was observed in 74% of patients. The median PFS and OS of the patient cohort were 318 days (10.4 months) and 898 days (29.4 months), respectively.

In the positive tissue control, fibulin-1 staining was mainly distributed throughout the idiopathic pulmonary fibrosis lung parenchyma and concentrated in cells within and around the alveolar space (Figure 7A) which is consistent with the findings in the study conducted by Jaffar et al. (184). Isotype control for all tissue sections had no non-specific staining (Figure 7B, D & F). Within the tumour sections, there was no observable staining for fibulin-1 in the malignant epithelial cells and positive staining was mainly localised within the stromal compartments, with staining intensities ranging from weak to strong for fibulin-1 (Figure 7C & E); this was also observed in adjacent sections of lymph nodes with tumours. Positive staining for fibulin-1 was observed in approximately 16% of patient samples in tumour sections, and in 25% of patients in adjacent lymph node sections (Table 7).


**Table 6. Patient characteristics and clinical outcomes in mCRC cohort examined in IHC**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14 (74)</td>
</tr>
<tr>
<td>Female</td>
<td>5 (26)</td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>60</td>
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<tr>
<td>Range</td>
<td>24 to 75</td>
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<tr>
<td><strong>ECOG PS</strong></td>
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<tr>
<td>0</td>
<td>15 (79)</td>
</tr>
<tr>
<td>1</td>
<td>4 (21)</td>
</tr>
<tr>
<td><strong>NLR</strong></td>
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</tr>
<tr>
<td>Median</td>
<td>2.8</td>
</tr>
<tr>
<td>Range</td>
<td>1.6 to 11.4</td>
</tr>
<tr>
<td>High (&gt;5)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Low (&lt;5)</td>
<td>15 (79)</td>
</tr>
<tr>
<td><strong>RECIST Response</strong></td>
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</tr>
<tr>
<td>Responsive (Complete/Partial)</td>
<td>10 (53)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>5 (26)</td>
</tr>
<tr>
<td><strong>PFS</strong></td>
<td></td>
</tr>
<tr>
<td>Median (days)</td>
<td>318</td>
</tr>
<tr>
<td>Range (days)</td>
<td>4 to 1964</td>
</tr>
<tr>
<td>PFS &lt; 1 year</td>
<td>10 (53)</td>
</tr>
<tr>
<td>PFS &gt; 1 year</td>
<td>9 (47)</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td></td>
</tr>
<tr>
<td>Median (days)</td>
<td>898</td>
</tr>
<tr>
<td>Range (days)</td>
<td>68 to 2027</td>
</tr>
<tr>
<td>OS &lt; 3 years</td>
<td>12 (63)</td>
</tr>
<tr>
<td>OS &gt; 3 years</td>
<td>7 (37)</td>
</tr>
</tbody>
</table>

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; NLR = Neutrophil/Lymphocyte ratio; RECIST = Response Evaluation Criteria In Solid Tumours; PFS = Progressive free survival; OS = Overall survival
Figure 7. Tissue fibulin-1 levels detected with IHC.
Representative images of human idiopathic pulmonary fibrosis lung parenchymal tissue (A – B) and mCRC tissue (C – F) staining for fibulin-1 (A, C & E) and isotype control (B, D & F). No non-specific staining was observed in the isotype controls (B, D & F). Fibulin-1 staining was observed throughout the stroma and concentrated in cells within the alveolar space (A). The malignant epithelial cells of the carcinoma have no positive staining for fibulin-1, whilst some tumour tissues show no (C) and strong staining (E) for fibulin-1 in the stroma surrounding the cancerous cells. Serial sections were used for staining fibulin-1 and isotype controls; however images were obtained randomly within the superficial tumour tissue. 100X magnification, scale bar represents 100μm.

Table 7. Proportion of tissue samples stained for fibulin-1 within the stroma by IHC.

<table>
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<tr>
<th></th>
<th>Negative staining (%)</th>
<th>Positive staining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining in tumour</td>
<td>16 (84.2)</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>Staining in lymph node</td>
<td>9 (75.0)</td>
<td>3 (25.0)</td>
</tr>
</tbody>
</table>
2.4.2.1 Stromal fibulin-1 expression in tumour tissue was not associated with chemotherapy response

In this cohort, patients were categorised into three response groups: responsive (partial and complete) (n = 10), stable disease (n = 4) and progressive disease (n = 5). There was no difference in fibulin-1 expression levels in the stroma of tumour tissue according to chemotherapy response (p value = 1.00) (Figure 8).

![Figure 8](image.png)

Figure 8. Analysis of stromal fibulin-1 staining in tumour tissue according to chemotherapy response. Stromal fibulin-1 expression in tumours was not significantly associated with chemotherapy response.

2.4.2.2 Stromal fibulin-1 expression in tumour does not predict clinical outcomes

Clinical outcomes that were analysed in this cohort were PFS and OS, these survival outcomes were dichotomised at 1 year and 3 years, respectively (n = 10 with PFS < 1 year and n = 9 with PFS > 1 year; n = 12 with OS < 3 year and n = 7 with OS > 3 year). A reduction in positive staining for stromal fibulin-1 was observed in patients with poorer survival; however, no significant associations were found between fibulin-1 expression and PFS or OS (p = 0.09 and p = 0.52, respectively) (Figure 9).
Figure 9. Analysis of fibulin-1 staining in stroma of tumour tissue with survival outcomes. Stromal fibulin-1 expression in tumours was not significantly associated with PFS (A) or OS (B).

2.4.3 Proteomic analysis of plasma fibulin-1

The use of SRM-MS to detect plasma fibulin-1 allows us to relatively quantify up to hundreds of peptides in a single multiplexed assay due to its high sensitivity, dynamic range, specificity and precision within a single sample injection (188). By doing so, we are able to selectively analyse a wider range of peptides believed to be representative of the expression of fibulin-1 in a complex biological sample such as plasma, instead of having to rely on and screen a variety of antibodies for each protein of interest as indicated by ELISAs.

To confirm and quantitate the expression of fibulin-1 in this patient cohort, targeted SRM-MS proteomic experiments were conducted and transitions for fibulin-1, CRP and SAA1 were optimised, the latter two proteins served as positive controls for systemic inflammatory response.

Examining extracted ion chromatograms (XIC) and searching MS/MS spectra against SwissProt databases confirmed the specificity of all transitions towards the peptide and protein of interest. Representative XIC and the MS/MS spectrum for peptides TGYYFDGISR (fibulin-1) (Figure 10A), SQETGDLDVGLQETDK (fibulin-1) (Figure 10B), ESDTSYVSLK (CRP) (Figure 11A) and SFFSFLGEAFDGAR (SAA1) (Figure 11B), all show the detection of fragment ions in the MS/MS spectrum (insert) and an overlap of these transitions in the XIC. The corresponding coloured arrows indicated in each MS/MS spectra point to at least two of the fragment ions.
Chapter 2 Investigation of plasma fibulin-1 as a prognostic marker in mCRC patients

(\(m/z\) range 300 – 1000) chosen to be targeted with SRM-MS for each peptide sequence; the fragment ions show an overlap of the transition signals in the XIC which indicate that they are specific for each peptide of interest.
Figure 10. Representative XICs and MS/MS spectras for two fibulin-1 peptides (TGYYFDGISR and SQETGDLDVGLQETDK). An overlap of the targeted transitions observed in the XICs of the peptide TGYYFDGISR (A) and SQETGDLDVGLQETDK (B) and the detection of these transitions indicated by the corresponding coloured arrows in each MS/MS spectra (inserts) reveal that the transitions are specific for the targeted peptide.
Figure 11. Representative XICs and MS/MS spectras for a peptide of CRP (ESDTSYVSLK) and SAA1 (SFFSFLGEAFDGAR).
An overlap of the targeted transitions observed in the XICs of the peptide of CRP, ESDTSYVSLK (A) and SAA1, SFFSFLGEAFDGAR (B) and the detection of these transitions indicated by the corresponding coloured arrows in each MS/MS spectra (inserts) reveal that the transitions are specific for the targeted peptide.
All targeted transitions signals had a high signal-to-noise ratio of > 10 which ensured reliable detection and quantitation of the transitions and they were detected and quantitated with reasonable reproducibility amongst the validating cohort, considering the signal intensities of these transitions were relatively low (coefficient of variance, CV ~20%) (Table 8). For each patient, signal intensity levels of each transition were plotted for the peptide of interest (raw data are found in Supplemental Table 1). Data for patients 5018, 5027 and 5057 were excluded from further analysis as the CVs for most of the transitions quantified were > 50%.

Table 8. SRM-MS reproducibility of targeted peptides across plasma samples from validating cohort.

<table>
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<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Q1 m/z</th>
<th>Q3 m/z</th>
<th>Av CV (%)</th>
<th>CV range (%)</th>
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<tbody>
<tr>
<td>Fibulin-1</td>
<td>TGYYFDGISR</td>
<td>589.78</td>
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<td>SQETGDLDVGGLQETDK</td>
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<td>847.42</td>
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<td>2.81 – 63.83</td>
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<tr>
<td>C-reactive protein</td>
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<td>Serum amyloid A1</td>
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<td>1082.53</td>
<td>25.00</td>
<td>3.47 – 74.93</td>
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</table>

As expected, patients with high NLR had increased expression of CRP and SAA1 with SRM-MS analysis. However, these relationships were not statistically significant due to high biological variability and low numbers of patients with high NLR (p = 0.60 and p = 0.41, respectively, (Figure 12A & B). Despite the high biological variability with SRM-MS, the CRP peptide detected with SRM-MS was significantly correlated with CRP clinical measurements in this cohort (r = 0.62 and p < 0.001) (Figure 12C). These results demonstrate that this proteomic technique can detect clinically relevant changes in inflammatory proteins.
Figure 12. SRM-MS analysis of CRP and SAA1 according to NLR and correlation of CRP. Patients with elevated systemic inflammation had elevated median levels of CRP (A) and SAA1 (B). CRP, peptide ESDTSYVSLK, analysed with SRM-MS was significantly correlated with clinically measured CRP (C).

2.4.4 Lack of correlation between tissue and plasma fibulin-1 expression

Next, I investigated whether plasma fibulin-1 was associated with stromal fibulin-1 expression in the tumour by examining the relationships between stromal fibulin-1 expression detected with IHC and plasma fibulin-1 detected in SRM-MS. Positive staining of fibulin-1 in the tumour stroma was observed in approximately 16% of patients. This reduction of stromal fibulin-1 expression within tumour tissue was not associated with the expression levels of plasma fibulin-1 peptides TGYYFDGISR and SQETGDLDVGLQETDK (p = 0.58 and p = 0.21, respectively) (Figure 13). Therefore, the extent of plasma fibulin-1 was not dependant on the amount of fibulin-1 deposited into the stroma in the cohort of patients analysed in IHC.
Figure 13. Tissue stromal and plasma fibulin-1 expression in mCRC patients. The level of fibulin-1 detected in the stroma of tumour tissue was not associated with the levels of plasma fibulin-1, expressed as TGYYFDGISR (A) and SQETGDLVGLQETDK (B) peptides.

2.4.5 Association of fibulin-1 detected with SRM-MS with NLR

A significant reduction of plasma fibulin-1 expression was observed for the TGYYFDGISR peptide in patients with high NLR (p = 0.04) (Figure 14A). However, this trend was not observed for the SQETGDLVGLQETDK peptide, where expression levels were similar irrespective of NLR (p = 0.60) (Figure 14B).

Figure 14. SRM-MS analysis of fibulin-1 according to NLR. Patients with elevated systemic inflammation had a reduction of fibulin-1 for only the TGYYFDGISR peptide (A) and not the SQETGDLVGLQETDK (B).
2.4.6 Evaluating the prognostic value of plasma fibulin-1 detected with proteomics with clinical outcomes

2.4.6.1 Plasma fibulin-1 expression does not predict chemotherapy response

Patients were categorised into three response groups: responsive (partial and complete) \( (n = 19) \), stable disease \( (n = 6) \) and progressive disease \( (n = 5) \). Non-parametric Kruskal-Wallis Test was conducted to determine whether fibulin-1 expression could predict for chemotherapy response. There was no difference in fibulin-1 expression levels for both peptides according to response \( (p = 0.31 \) and \( p = 0.60 \) for TGYYFDGISR and SQETGDLDVGGLQETDK, respectively) (Figure 15).

![Figure 15](image)

Figure 15. SRM-MS analysis of fibulin-1 expression according to chemotherapy. Expression of both fibulin-1 TGYYFDGISR (A) and SQETGDLDVGGLQETDK (B) peptides were not associated to response.

2.4.6.2 Plasma fibulin-1 expression predicts survival outcomes

I also investigated the prognostic value of fibulin-1 in this cohort. PFS was dichotomised at 1 year for analysis \( (n = 22 \) with PFS < 1 year and \( n = 10 \) with PFS > 1 year). There was no significant difference in expression of either peptide according to 1 year PFS \( (p = 0.71 \) and \( p = 0.95 \) for TGYYFDGISR and SQETGDLDVGGLQETDK, respectively) (Figure 16A & B).
Figure 16. SRM-MS analysis of fibulin-1 expression according to PFS. Fibulin-1 peptides, TGYYFDGISR (A) and SQETGDLVGLQETDK (B) were not associated with PFS when dichotomised at 1 year.

A reduction of fibulin-1 was observed to be associated with shorter OS. This was consistent when OS was dichotomised at 3 years (n = 21 with OS < 3 years and n = 11 with OS > 3 years) and as a continuous variable (Figure 17A – D). However, only the TGYYFDGISR peptide was significantly associated with OS when defined with a cut-off of 3 years and as a continuous value (p = 0.001 and p = 0.01, respectively) (Figure 17A & C); whilst the SQETGDLVGLQETDK peptide showed the same trend but did not reach statistical significance when OS was analysed with a 3 year cut off and as a continuous value (p = 0.08 and p = 0.11, respectively) (Figure 17B & D). Therefore TGYYFDGISR peptide shows promising evidence of being a suitable prognostic marker for OS in patients with mCRC.
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Figure 17. SRM-MS analysis of fibulin-1 expression according to OS. A reduction in fibulin-1 was observed to be associated with poor OS in both TGYYFDGISR and SQETGDLDVGLQETDK peptides when OS was dichotomised at 3 years (A – B) and categorised as a continuous value (C – D). However, only the TGYYFDGISR peptide was significant.

2.4.7 Validating SRM-MS results of plasma fibulin-1 expression with Western blotting

Validation of biomarkers require the use of traditional protein assays, such as Western blotting and ELISAs to confirm proteomic findings and obtain absolute reference values for use as a prognostic biomarker in a disease setting. I validated my SRM-MS proteomics results with Western blotting as the anti-human fibulin-1 ELISA kit available from USCNK cross reacts with plasma fibronectin (Jade Jaffar, Post-Doctorate Fellow, The Alfred Hospital and Monash University, personal communication).
I investigated whether validation with Western blotting could confirm the proteomic findings of reduced fibulin-1 being associated with poorer OS. A specific fibulin-1 monoclonal antibody (clone B-5, Santa Cruz) was used in these Western blotting experiments to measure fibulin-1 expression in plasma samples of the validating cohort.

Three bands for fibulin-1 were detected in all plasma samples; the mature fibulin-1 polypeptide (molecular mass of 100kDa) and two smaller fragments (molecular mass of 70 and 50kDa) (Figure 18).

**Figure 18.** 32 mCRC patients plasma samples prior to chemotherapy in a representative Western blot. Three immunogenic fibulin-1 bands (molecular mass of 100, 70 and 50kDa) were detected in plasma of mCRC patients. Abbreviations: L indicates the protein molecular weight ladder, FN is fibronectin (the negative control) and N ctrl is the normalising healthy plasma sample control.
2.4.7.1 No associations between NLR and three fibulin-1 fragment bands

According to NLR, there was no significant difference in expression levels with any of the three fibulin-1 fragments detected in plasma (p = 0.76, 0.52 and 0.41 for the 100, 70 and 50kDa fibulin-1 fragments, respectively) (Figure 19).

![Figure 19](image)

**Figure 19.** Western blotting analysis for fibulin-1 according to NLR. There were no distinct associations with the expression levels of the 100 (A), 70 (B) and 50kDa (C) fibulin-1 fragments according to NLR.

2.4.7.2 Fibulin-1 expression analysed with Western blotting does not predict response

Consistent with the SRM-MS proteomics findings, chemotherapy response was not associated with fibulin-1 expression. There was a trend towards reduced fibulin-1 expression in patients with stable disease for the 70kDa fragment (p = 0.23, 0.08 and 0.86 for 100, 70 and 50kDa fibulin-1 fragments, respectively) (Figure 20).
2.4.7.3 Plasma fibulin-1 expression analysed with Western blotting predicts overall survival

Surprisingly, I found a significant reduction of fibulin-1 expression for the 50kDa fragment was associated with PFS greater than 1 year (p = 0.03), this trend was also observed for the 100kDa fragment (p = 0.08) and the 70kDa fragment was not significantly associated with PFS (p = 0.53) (Figure 21).

Figure 20. Western blotting analysis for fibulin-1 according to chemotherapy response. Response to chemotherapy was not associated with the expression levels of the 100 (A), 70 (B) and 50kDa (C) fibulin-1 fragments.

Figure 21. Western blotting analysis for fibulin-1 according to PFS. A reduction of fibulin-1 expression was observed for the 100kDa (A), 70kDa (B) and 50kDa (C) fragment in patients with PFS > 1 year, however this association was only significant for the 50kDa fragment.
Consistent with the SRM-MS proteomics findings, the 70kDa fibulin-1 fragment was significantly reduced in patients with poorer OS using OS dichotomised at 3 years (p-value = 0.03, Figure 22B) and as a continuous value (p-value = 0.004, Figure 22E). There was no change to OS when dichotomised at 3 years for the other two fibulin-1 fragments (100 and 50kDa) (p = 0.70 and p = 0.94, respectively) (Figure 22A & C). Kaplan-Meier survival analysis of the other two fibulin-1 fragments (100 and 50kDa) revealed no significant differences according to OS (p = 0.87 and p = 0.18, respectively) (Figure 22D & F).
Figure 22. Western blot analysis of fibulin-1 according to OS. Only the 70kDa fragment (B) was significantly associated with OS when dichotomized at 3 years; the 100kDa (A) and 50kDa fragments had no correlation with OS. Kaplan-Meier survival analysis of fibulin-1 fragment 100kDa (D), 70kDa (E) and 50kDa (F) shows that patients with reduced expression of the 70kDa fibulin-1 fragment have significantly poorer OS.
2.4.8 Cytokine expression with fibulin-1 expression and clinical patient outcomes

To further understand the relationships between fibulin-1 and systemic inflammation and how cytokines may play a role in dictating clinical outcomes of mCRC patients, I measured the level of cytokines implicated in systemic inflammation, IL-6, IL-8 and CXCL1, using ELISAs.

Three patients had high CVs (CV > 30%) within duplicate samples for IL-6 and were removed from subsequent analysis. All but three patients (R09, R11 and R15) had their CRP concentrations clinically measured. Expression levels of pro-inflammatory mediators CRP, IL-6, IL-8 and CXCL1 were correlated with fibulin-1 expression analysed with SRM-MS and Western blotting experiments (averaged values are found in Table 9). The median signal intensity value for the fibulin-1 peptides targeted by SRM-MS, TGYFDGISR and SQETGDLDVGLQETDK, were $1.44 \times 10^5$ cps and $1.21 \times 10^5$ cps, respectively.
Table 9. Averaged expression levels of CRP, IL-6, IL-8, CXCL1 and FBLN1 (analysed with SRM-MS and Western blotting)

<table>
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<tr>
<th>Patient ID</th>
<th>CRP (μg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-8 (pg/mL)</th>
<th>CXCL1 (pg/mL)</th>
<th>TGYYFDGISR (cps)</th>
<th>SQETGDLVVGQ QTDK (cps)</th>
<th>WB-100kDa</th>
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<td>117631.79</td>
<td>1.39</td>
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^ These patient concentrations were excluded from further analysis due to high inter-replicate CVs (> 30%)
Patients with high NLR had significantly elevated levels of CRP ($p < 0.01$) and trending towards elevated levels of IL-6 and IL-8 ($p = 0.16$ and $p = 0.23$, respectively). There was no difference in CXCL1 expression between the two NLR groups (Figure 23A). In patients with lower than median levels of the TGYYFDGISR peptide, they were significantly associated with elevated expression levels of CRP and IL-8 ($p < 0.001$ and $p < 0.05$, respectively) and there was a trend for increased IL-6 concentration ($p = 0.07$). For the SQETGDLDVGGLQETDK peptide, reduced expression was only significantly associated with elevated IL-8 levels ($p = 0.03$) and again, trended with higher IL-6 concentration ($p = 0.20$). No significant changes for CXCL1 were detected for both the peptides ($p = 0.91$ and $p = 0.56$ for TGYYFDGISR and SQETGDLDVGGLQETDK, respectively) (Figure 23B & C). Unfortunately, these findings were not confirmed with the fibulin-1 fragments detected by Western blotting (Figure 24).
Figure 23. Analysis of inflammatory cytokines according to NLR and the two fibulin-1 peptides analysed in SRM-MS.

Patients with elevated systemic inflammation (NLR > 5) had elevated levels of CRP, IL-6 and IL-8 (although significance was only observed with CRP levels) (A). Patients with below median of fibulin-1 TGYYFDGISR peptide has significant elevated levels of CRP and IL-8 (a favourable trend for increased IL-6 levels was also observed) (B) and elevated IL-8 levels was only significantly correlated with the below median SQETGDLDVGLQETDK peptide levels (IL-6 was also observed to be increase but was not significant) (C). Expression levels of CXCL1 were not associated with either NLR or both fibulin-1 peptides.
Figure 24. Analysis of inflammatory cytokines according to the three fibulin-1 fragments analysed in Western blotting.
No associations were found between CRP, IL-6, IL-8 and CXCL1 for the 100kDa (A), 70kDa (B) and 50kDa (C) fibulin-1 fragments.
2.5 Discussion

Fibulin-1 is an ECM and plasma protein implicated in cancer (142, 151, 154, 155, 164, 179). It mediates cellular processes and ECM remodelling, which is crucial for regulating the behaviour of malignant cells during carcinogenesis. ECM remodelling is indicative of highly invasive and metastatic tumours and poorer patient survival. Altered tumour cellular expression of fibulin-1 has been reported in many cancer types. However, there is very little information regarding the roles of the various forms of fibulin-1 in CRC carcinogenesis and whether they have any prognostic values in predicting clinical outcomes.

In this Chapter, fibulin-1 was not detected in malignant CRC cells in tumour tissue. Reduced levels of fibulin-1 were observed in the stromal compartments of tumour tissue. There was no correlation between the expression levels of secreted plasma and deposited fibulin-1. The prognostic value of fibulin-1 was only observed for plasma fibulin-1 in mCRC patients, where a reduction was associated with poorer OS. A reduction in plasma fibulin-1 levels was also found to be associated with elevated systemic inflammation.

2.5.1 Tumoural and plasma fibulin-1 expression in mCRC patients

Within the tumour tissue of the mCRC cohort, fibulin-1 was not detected in the cancerous cells but its expression was observed in the stromal compartment in some patient samples. This finding is consistent with the studies conducted by Wen et al. and Roark et al. where it was found that fibulin-1 expression was non-existent in malignant colonic epithelial cells and the expression of fibulin-1 in the stroma was reduced in colorectal carcinoma tissue in comparison to adjacent normal colon mucosal tissue and adenocarcinomas (139, 153). I did not have access to adjacent normal tissue, therefore was unable to determine whether the expression of fibulin-1 in these samples were lower than normal tissue.
Not only is fibulin-1 a component of the ECM, but it can also be secreted into the circulation (135). Currently, there is no information regarding the association between secreted expression of fibulin-1 with ECM-embedded fibulin-1 expression in mCRC patients. I found no associations between these two forms of fibulin-1 within the patient cohort; that is, the extent of fibulin-1 observed in CRC stroma was not dependent on the levels of secreted fibulin-1. It is possible that there are mechanisms involved in regulating the expression of either forms that are independent of one another or the source of secreted fibulin-1 does not arise from the tumour cells themselves but from other supporting stromal cells outside of the tumour microenvironment. However, increased plasma fibulin-1 has also been associated with diabetes, renal impairment, airway and cardiovascular diseases (132, 133, 184-186); therefore, comorbidities may be confounding my findings, and I cannot rule out the impact of these comorbidities as I did not have this information about the patient cohort.

The study by Sadlonova et al. investigated molecular differences between normal fibroblasts derived from normal breast tissue and cancer-associated fibroblasts (CAFs) derived from breast carcinomas and found mRNA expression of fibulin-1 to be downregulated in CAFs (195). CAFs are one of the most abundant stromal cells found within the tumour microenvironment and generally promote tumour growth by initiating proliferation, inflammation, angiogenesis, and metastasis (196). Therefore, it is possible that the stromal fibulin-1 detected in some samples in the IHC experiments were from both the ECM and fibroblasts; and a reduction in this expression could be due to the involvement of CAFs within the tumour. The involvement of CAFs at later stages of tumour progression is beneficial for the evolving tumour as CAFs secrete pro-inflammatory cytokines and growth factors such as IL-6, IL-8 and VEGF, as well as alter ECM composition, which in turns influences the fate of a malignant cell and can dictate patient outcomes during carcinogenesis (195-197). Fibulin-1 is also deposited throughout the layers of the arterial walls, but predominately expressed in the matrix that surrounds the vascular smooth muscles and in the elastic lamina (132). It is here that fibulin-1 binds with
elastin to provide structural integrity to elastic fibres to maintain and stabilise vessel formation during angiogenesis (139). Therefore, in more advanced CRCs it is possible that fibulin-1 may be retained in blood vessels to assist in angiogenesis and is not easily secreted into the circulation which may account for why I observed a reduction in plasma fibulin-1 in patients with poorer survival.

2.5.2 Prognostic value of tumour and plasma fibulin-1 expression

With previous reports of increasing tumour grade being associated with poorer survival as well as a reduction of fibulin-1 in CRC patients, I wanted to determine whether tumour or plasma fibulin-1 expression predicts chemotherapy response and clinical outcomes in mCRC patients.

2.5.2.1 Chemotherapy response

There was no association of stromal and plasma fibulin-1 in predicting chemotherapy response in mCRC patients. Neither stromal or plasma fibulin-1 was predictive of PFS. This is likely because PFS is also a surrogate marker of response; it serves to evaluate how effective a cancer treatment is (198). Therefore, it was not surprising that fibulin-1 expression was not associated with PFS, as there was no association between fibulin-1 expression and chemotherapy response rate. However, the study conducted by Pupa et al. in breast cancer has found that fibulin-1 expression can regulate chemotherapy response and protected cancer cells from chemotherapy-induced apoptosis (180). The mechanisms for chemotherapy resistance by fibulin-1 in breast cancer are unknown. However, it has been suggested to be mediated through β1 integrin receptor signalling by activating PI3-kinase/AKT and MAPK/ERK survival signalling pathways to inhibit apoptosis (180, 199). Changes to the expression profiles of ECM proteins (such as collagen, laminin, fibronectin, integrins and fibulin-1), MMPs and TIMPs, which can lead to ECM remodelling and enhanced tumourigenicity, have been found to be associated with chemo-resistant cancer cell lines and tumour tissue (200-203).
2.5.2.2 Survival

In my studies, a reduction of positive staining of stromal fibulin-1 expression within tumour sections was observed in patients with poorer OS. Despite this finding not being significant, the finding is consistent with other non-oestrogen sensitive cancers such as hepatocellular, cutaneous melanoma and bladder cancer which have investigated the prognostic value of fibulin-1; in these cancers, a reduction of fibulin-1 expression (due to promoter methylation) in tumour tissue was associated with poorer survival (147-149).

I evaluated the prognostic value of fibulin-1 and found that a reduction in plasma fibulin-1 levels analysed with SRM-MS and Western blotting was significantly associated with poorer OS in mCRC patients. To my knowledge, this is the first time plasma fibulin-1 has been found to be associated with predicting survival outcomes in cancer patients.

Extensive research has investigated the functional role of cellular fibulin-1 expression in several cancer types. Overexpression of cellular fibulin-1 in cell lines of fibrosarcoma, melanoma, prostate, renal, breast and ovarian cancer, has been found to be associated with increased apoptosis, reduced cellular proliferation, motility, invasion and angiogenesis in vitro (142, 151, 154, 161, 179). These results demonstrate that decreased cellular fibulin-1 expression enhances the ability of malignant cells to develop into an aggressive cancer phenotype. This could provide a possible explanation for my clinical findings that reduced plasma fibulin-1 results in poorer OS in mCRC patients. However, the mechanisms of how plasma fibulin-1 reflects the carcinogenic processes of cellular proliferation, motility, invasion and angiogenesis are still not well understood and have yet to be studied in CRC.

2.5.3 Relationship between systemic inflammation and fibulin-1 expression.

Systemic inflammation plays a vital role in influencing the carcinogenic process and over the years, has served as a prognostic marker in advanced stages of cancer (103, 113). I found that
in this small independent cohort of patients, that patients with elevated systemic inflammation, determined as NLR > 5, had elevated levels of pro-inflammatory cytokines (CRP, IL-6 and IL-8), no clinical benefit from chemotherapy and poorer survival compared to patients with low circulating immune cell levels. This is consistent with the study conducted by Chua et al. (104), which confirmed that NLR, a surrogate marker of systemic inflammatory response, can be used to predict clinical benefit from chemotherapy and survival in mCRC patients.

In this study, SRM-MS proteomic analysis of the targeted peptides revealed that a reduced level of fibulin-1 using the TGYYFDGISR peptide was associated with elevated systemic inflammation, pro-inflammatory cytokines and poorer OS. It is still unsure why the TGYYFDGISR peptide, and not the SQETGDLDVGLQETDK peptide, is associated with OS. It has been found that fibulin-1, as well as other members of the fibulin family, are readily cleaved and highly susceptible to proteolysis within the N-terminus region (204, 205). The peptide SQETGDLDVGLQETDK is located within the N-terminus whilst TGYYFDGISR is present within the central domain of the protein; therefore, it could be possible that the level of proteolysis within the two domains may affect the expression levels of these peptide sequences detected with SRM-MS and subsequently its correlation with clinical outcomes. Further validation using Western blotting confirmed the prognostic utility of plasma fibulin-1 to predict OS in patients with mCRC. This is the first time, to my knowledge, that plasma fibulin-1 has been linked to both systemic inflammation and clinical outcomes; suggesting circulating plasma fibulin-1 concentrations may be influenced by the inflammatory response in patients with mCRC or vice versa. To further highlight the importance of secreted ECM proteins in carcinogenesis, increasing studies have recently associated secreted ECM proteins with systemic inflammation and clinical outcomes (125-127, 129, 206-209).

Levels of serum endostatin, a protein fragment of collagen type XVIII cleaved by several MMPs that acts as an angiogenesis inhibitor, were elevated in CRC patients in comparison to age-
sex- matched healthy controls (206). Elevated levels of endostatin were significantly correlated with elevated systemic inflammatory markers such as CRP, NLR and modified GPS; increasing tumour stage and invasion through the muscularis propria and there was a trend for an association with poorer OS (206). The study conducted by Sun et al. in colon cancer patients found elevated serum fibrinogen levels were positively correlated with systemic inflammatory markers, modified GPS, NLR and platelet-lymphocyte ratio (PLR); tumour grade and size and negatively associated with poorer OS (125). MMPs and TIMPs play an important role in regulating ECM dynamics, the expression of MMPs and TIMPs have been found to be increased by pro- and anti-inflammatory cytokines, respectively, and produced from either malignant cells or tumour-associated immune cells (77). Therefore, it comes as no surprise that they are associated with predicting patient outcomes in cancer, for example, serum MMP-2, 7, 8, 9, 13 and TIMP-1,2 are associated with poor prognosis in advanced CRC patients (129, 130, 208-211). Although the mechanisms linking systemic inflammation and ECM proteins is unclear, these studies highlight the crucial role inflammation plays in influencing the secretion of ECM proteins which affects cellular behaviour and initiates ECM remodelling during carcinogenesis to increase tumour growth, invasion and metastasis which ultimately dictates patient prognosis.

In CRC cancer, there is little information about the regulation of fibulin-1 and role in cancer progression. Fibulin-1 mRNA and protein cellular expression is reduced in CRC cell lines and human CRC tissue specimens in comparison to normal colon cell lines and tissue (153). Unlike other cancers, the reduction of fibulin-1 expression is not due to hypermethylation or loss of heterozygosity, but due to transcriptional repression. Tumour cellular fibulin-1 expression has been shown to be downregulated by the inflammatory chemokine, CXCL1, which is over expressed in CRC (147-150, 153). Unfortunately, in this study, I found no associations between serum CXCL1 and plasma fibulin-1 expression levels. This simply could suggest that cellular CXCL1 is a more potent regulator of tumour/stroma fibulin-1 cellular expression in comparison
to the secreted form. However, I did find that plasma fibulin-1 expression was associated with plasma IL-8 and weakly with plasma IL-6 concentrations.

Similarly to IL-6, IL-8 plays a crucial role in carcinogenesis, mainly to promote the recruitment of neutrophils into the tumour microenvironment and to increase cellular proliferation, survival and migration of endothelial cells involved in the angiogenic response (212). Regulation of IL-8 expression can be through NF-κB-activation, as well as inflammatory stimuli such as IL-1β, tumour necrosis factor α, and IL-6, exposure to chemotherapy agents and environmental stresses such as hypoxia, and steroid hormones. Therefore, additional studies are required to further understand the mechanisms that inflammatory cytokines, particularly IL-6 and IL-8, use to regulate the secreted version of fibulin-1.

2.5.4 Technical considerations

There were several limitations to this study which may have hindered unmasking other important relationships between systemic inflammation and clinical outcomes. I acknowledge my small cohort size may have contributed to the large biological variability observed in the results for many of the IHC, SRM-MS and cytokine assays. Future studies need to be conducted in a larger cohort to independently reproduce and confirm these results.

Accurate quantitation of proteins in complex biological specimens such as plasma continues to be a challenging aspect of clinical cancer biomarker proteomics. There have been many studies that have shown improvements of the detection of lower abundance proteins after the depletion of high abundance proteins (191, 213, 214). However, only recently, the depletion of high abundance plasma proteins has been found to be counter-productive in the quantitation of lower abundance proteins. A loss of 27 cytokines analysed with a multiplex ELISA kit by Ahn et al. (215) was observed in both fractions (flow through and bound) of the depleted plasma samples compared to neat plasma in healthy volunteers; this could be due to a combination of post-depletion precipitation of proteins and/or resuspended of pellets and the non-specific
removal of the lower abundant proteins during the depletion process. Targeting low abundant proteins bordering the lower detection limit (approximately $10^4$ cps) such as fibulin-1, without fractionation, increases the signal-to-noise ratio and compromises the high quality detection for SRM-MS (188). To further improve the detection of these lower abundance proteins with SRM-MS, without having the need of immunoaffinity depletion or enrichment procedures, spiking plasma samples with exogenous stable isotope-labelled standard peptides has been shown to detect proteins of concentrations as low as ng/mL with high reproducibility, signal stability and sensitivity (216). The concentration of plasma fibulin-1 in healthy individuals is in the μg/mL concentration range, which is promising; however, proteomic methods need to be optimised to enhance the detection this lower abundant protein (132, 133).

Western blotting confirmed the prognostic value of fibulin-1 in predicting OS in mCRC patients in the 70kDa fragment. It should be noted that the epitope region for the fibulin-1 antibody used corresponded to the sequence of amino acids 1 – 190, which only aligns with the SQETGDLDVGGGLQETDK peptide. This could possibly explain the discrepancy and lack of correlation between the SRM-MS and Western blotting results. Also, it is not known what the amino acid sequences are for each of the three fragment bands detected in the plasma by Western blotting in this cohort. Despite the overlap of the SQETGDLDVGGGLQETDK peptide sequence with the monoclonal antibody used in Western blotting, the antibody used may have detected other sequences of fibulin-1 for the three fragment band; therefore, the 70kDa fragment band may have arisen from another sequence that may be important for predicting clinical outcomes, and either one (or none) of the two other fragments may contain the SQETGDLDVGGGLQETDK sequence. Further research into validating the prognostic value of fibulin-1 needs to incorporate the peptide sequence TGYYFDGISR as an immunogen and determining its position in the intact, full length fibulin-1 protein for antibody development.
2.5.5 Conclusions

In this chapter, it was found that a reduction of plasma fibulin-1 was associated with elevated plasma cytokines and poorer OS in mCRC patients. This study provided two novel findings; 1) new evidence demonstrating a relationship between systemic inflammation and secreted fibulin-1 expression and 2) the utility of fibulin-1 as a prognostic marker of OS in patients with mCRC. Further studies are required to elucidate the regulatory relationships between pro-inflammatory cytokines and the different forms of fibulin-1. This information may provide beneficial mechanisms to understand the correlation between reduced plasma fibulin-1 expression and poorer survival.
Chapter 3

*In vitro* investigation of fibulin-1 expression and functional role in colorectal cancer.
3.1 Introduction

In Chapter 2, I found that cellular expression of fibulin-1 was limited to low expression only in stromal cells of tumour tissue in a small number of patients and was not associated with patient outcomes. However, reduced plasma fibulin-1 expression was significantly associated with elevated levels of pro-inflammatory mediators and poorer OS in this cohort of mCRC patients. Pro-inflammatory cytokines, such as IL-1, IL-6, and TNF are crucial mediators of CRC carcinogenesis (217). It is known that elevated levels of IL-6 are associated with advance tumour stage and poor survival in CRC patients (218, 219). Increased IL-6 levels can initiate the production of cytokines, chemokines, proliferative and angiogenic growth factors and ECM proteins to promote immunosuppression, tumour growth, angiogenesis and ECM remodelling (217). Elevated pro-inflammatory cytokines can regulate the expression of ECM proteins to degrade the ECM to facilitate in the invasion and spread of malignant cells as well as increase the tumour’s access to the vasculature system. IL-6 has been found to induce MMP-9 expression in macrophages in an increasing manner (220). MMP-9 is one of the many MMPs found to be overexpressed in CRC, where increasing serum MMP-9 expression has also been positively correlated with elevated IL-8 (221, 222). Other pro-inflammatory cytokines, such as TNF and interferon-γ, have also been found to increase the secretion of ECM protein, laminin, in human intestinal epithelial cells which has been found to mediate cellular adhesion, proliferation, migration and differentiation in vitro (223). Identifying the inflammatory proteins that regulate fibulin-1 expression in CRC patients may provide a way to reverse the reduction in fibulin-1 observed in many tumour types and improve patient outcomes.

There are limited studies that have investigated the regulation of fibulin-1 expression in cancer. Promoter hypermethylation has been shown to downregulate tissue fibulin-1 expression in hepatocellular, cutaneous melanoma and bladder cancer (147-149). However, in advanced CRC, promoter hypermethylation was not observed and an overexpression of CXCL1 was found to transcriptionally downregulate cellular fibulin-1 expression in tumour cell lines.
and promote tumour cell proliferation \textit{in vitro} (153). Silencing CXCL1 in HCT-15 CRC cells increased fibulin-1 expression leading to increased cellular apoptosis and inhibition of cellular proliferation of CRC cells (153). CXCL1 is expressed by myeloid cells, CAFs and epithelial cells in tumours and interacts with CXCR2 to promote proliferation and migration of CRC tumour cells, endothelial cells, myeloid-derived suppressor cells and neutrophils (91, 224-226). A similar relationship between increased CXCL1 reducing tumoural fibulin-1 expression has also been confirmed in prostate cancer (154). Under inflammatory conditions, CXCL1 is upregulated via the activation of NF-\(\kappa\)B and AKT pathways. Numerous cytokines and growth factors, such as TNF, IL-1\(\beta\), IL-17 and IL-6, that are upregulated in the CRC tumour microenvironment can further activate NF-\(\kappa\)B and AKT signalling pathways, via STAT-3 activation (217). However, none of these cytokines have been investigated in the regulation of the secreted version of fibulin-1.

Secreted fibulin-1 expression has also been found to be regulated by MMP-13 (157). Through MMP-13-mediated proteolysis, secreted fibulin-1 expression was decreased and led to enhanced cell migration in HeLa cells (157). In CRC tumours, MMP-13 is over-expressed and associated with tumour invasion, metastasis, and poorer prognosis in CRC patients (158, 159, 210). MMP-13 is expressed by many different cell types such as epithelial cells, endothelial cells, fibroblasts, as an inactive proform which requires activation through the proteolysis of other MMPs. MMP-13 is regulated by a range of growth factors and cytokines including IL-1, IL-6, TNF and insulin-like growth factor (159, 227, 228). Thus, one or more of these inflammatory mediators may contribute to increased tumoural expression of CXCL1 and MMP-13 and repression of fibulin-1 in CRC tumours.

The ability of fibulin-1 to influence cell motility and adhesion is due to fibulin-1 binding to and interacting with various ECM proteins such as fibronectin, laminin, aggrecan and versican. Fibulin-1 has been found to inhibit fibronectin-regulated cell attachment and motility in human
Chapter 3 In vitro investigation of fibulin-1 expression and functional role in colorectal cancer

breast cancer cell lines (161). As most cancers have reduced expression of fibulin-1, the inhibitory effect of fibulin-1 on cell adhesion and motility is reduced, allowing for cancerous cells to invade the ECM with greater ease. Furthermore, by binding to and interacting with angiogenin and endostatin, a pro- and anti-angiogenic agent, respectively, suggests that fibulin-1 may play a role in regulating angiogenesis to promote carcinogenesis (164, 171).

However, the mechanisms by which both of these ECM proteins in the presence of fibulin-1 regulate angiogenesis are still unknown. The study by Xiao et al, found that overexpression of fibulin-1 in CM of ACHN renal cancer cells resulted in a reduction in tube formation, which was restored with the treatment of fibulin-1 antibody in vitro, and tumour sections in nude mice staining for the specific endothelial cell specific marker CD31 had reduced blood vessels in vivo (151). In HT1080 fibrosarcoma cell lines, increased genetic expression of cellular fibulin-1 suppressed tumour growth and angiogenesis in vivo (179). Also, cathepsin D, a tumour microenvironment-associated protease similar to MMPs, degrades fibulin-1 to produce a small fragment named neostatin. This neostatin fragment was found to inhibit endothelial proliferation and tube formation in vitro (179). These studies provide evidence that fibulin-1 may function as an angiogenesis inhibitor.

In CRC, there is no information regarding the relationships between cellular, secreted and ECM embedded fibulin-1 expression. The regulation of these three protein forms of fibulin-1 and the functional role of secreted fibulin-1 in CRC carcinogenesis is still unknown. Investigating the expression and regulatory mechanisms of all three fibulin-1 forms will be essential in understanding the source and role of this protein in tumour progression. Examination of these factors under inflammatory conditions will also provide insights into mechanisms regulating the reduction of plasma fibulin-1 and poorer OS in mCRC patients with elevated systemic inflammation.
Chapter 3 In vitro investigation of fibulin-1 expression and functional role in colorectal cancer

3.2 Aims

The aims for this chapter were:

1. To examine the expression of all three forms of fibulin-1, CXCL1, CXCR2 and MMP-13 in human colonic fibroblast and CRC cell lines.

2. To elucidate the role of CXCL1 and MMP13 on fibulin-1 expression in human colonic fibroblast and CRC cell lines.

3. To investigate the functional role of fibulin-1 on migration and angiogenesis during carcinogenesis.

4. To determine whether fibulin-1, CXCL1, CXCR2 and MMP-13 expression and functional responses are changed under IL-6 treatment.
Chapter 3 In vitro investigation of fibulin-1 expression and functional role in colorectal cancer

3.3 Methods

3.3.1 Cell culture

Six human colon adenocarcinoma cell lines LIM2405, Caco-2, HT29, HCT116, SW48, and SW620, human colonic fibroblast cell line CCD-18Co and human umbilical vein endothelial cells (HUVEC) were used in these in vitro studies. CCD-18Co fibroblast, SW48, SW620, HCT116, and HT29 were cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies); Caco-2 was cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and 1% non-essential amino acids (Life Technologies); LIM2405 was cultured in RPMI-160 medium supplemented with 10% FBS, 1 µg/mL hydrocortisone, 10 µM 1-thioglycerol, 0.6 µg/mL insulin and 25 mM HEPES (Life Technologies) and HUVEC was cultured in Media 200PRF supplemented with low serum growth supplement. All cell lines were incubated in a humidified atmosphere of 37°C in 5% CO₂. Only passages 2 to 6 for HUVECs; and a maximum of 20 passages for CCD-18Co fibroblast and CRC cell lines were used in these experiments.

All CRC and fibroblast cells were seeded in single wells in 6-well plates (for RNA and protein analysis) and in 12 replicates in 96-well plates (for ECM ELISA) according to the seeding densities listed in Table 10 in growth media for 72 hours until they reached approximately 75% confluency. After 72 hours, cells were quiesced in their respective culture media supplement with 0.1% FBS (quiescing medium) for 24 hours and then treated with or without 10 ng/ml IL-6 in quiescing medium for a further 72 hours. RNA and protein samples were collected and stored at -80°C (RNA) and -20°C (supernatant and protein) until analysis.
### Table 10. Seeding densities for six human colon adenocarcinoma and fibroblast cell lines.

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<th>Wound scratch assay</th>
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#### 3.3.2 Protein extraction, concentration and determination

To determine the protein expression of cellular and secreted fibulin-1 and CXCL1, human colon fibroblast and CRC cells were seeded in 6-well plates and treated with or without IL-6 as described above. Supernatant was collected and spun at 16,000g at 4°C for 5 minutes to remove cellular debris. Total cellular protein was extracted by using lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and cOmplete protease inhibitor cocktail, mini (Roche). Cell lysates were collected by scraping the cells off the plates with chilled lysis buffer and a cold plastic cell scraper (Greiner Bio-One), cell suspension was then constantly agitated at 4°C for 30 minutes with a tube rotator before centrifuging at 16,000g at 4°C for 20 minutes to pellet cellular debris. Cell lysate and supernatant samples were concentrated by freeze drying the samples overnight with a FreeZone -105°C 4.5L cascade bench top freeze dry system (Labconco). Freeze dried samples were reconstituted with 200 μL of MilliQ water.

Total protein was determined by the Pierce BCA protein assay (Thermo Scientific) according to the manufacture’s protocol. Briefly, protein concentration in cell lysate and supernatant samples was measured using a colourmetric protein assay based on a shift in absorbance of BCA upon binding to protein. Cell culture samples were diluted (1:10) with MilliQ water and 25 μL of this diluted sample was added to 200 μL BCA working reagent and incubated at room temperature for 30 minutes. Samples were quantitated in duplicate using a bovine serum
albumin as a protein standard (0 – 2 mg/mL) and absorbance was measured with a FLUOstar Omega spectrophotometer (BMG Labtech) at 562 nm.

3.3.3 Western blotting

3.3.3.1 Preparation of methanol-free Coomassie staining protocol

Coomassie Brilliant Blue (G-250) (60 mg) was dissolved in 1 L distilled water by stirring for 2 – 4 hours over a magnetic hotplate before the addition of 6 mL of HCl and stored at room temperature, covered.

3.3.3.2 Gel electrophoresis

Soluble and cellular fibulin-1 were detected by Western blotting (194) with 1.2 µg of cell lysate and 12 µg of supernatant loaded in each well and each gel loaded with a positive (1% FBS), negative (100 ng fibronectin) and normalising (1:500 healthy plasma sample) control. Samples were aliquoted into working loading volumes to ensure the number of freeze thaw cycles for the samples do not exceed three cycles. Proteins were separated on 10% SDS polyacrylamide gels at 125 V for 70 minutes, transferred to PVDF membranes at 25 V for 1.5 hours and blocked with 5% skim milk/2% BSA/TBS/0.1% Tween-20 blocking solution at room temperature for 1 hour. All antibodies were diluted in 2% BSA/TBS/0.1% Tween-20. Membranes were incubated with mouse anti-human fibulin-1 monoclonal antibody (clone B-5) (Santa Cruz, 0.2 µg/mL for supernatant samples and 0.3 µg/mL for cell lysate samples) at 4°C overnight. Following five 3 minute TBS/0.1% Tween-20 washes, membranes were then incubated with goat anti-mouse IgG-HRP polyclonal antibody (DAKO, 0.2 µg/mL for supernatant samples and 0.3 µg/mL for cell lysate samples) at room temperature for 1 hour. After washing, blots were visualised and exposed with Immobilon Western Chemiluminescent HRP substrate (Millipore) for 3 minutes, bands were analysed using ChemiDoc™ MP Imaging system (Bio-Rad), and the amount of protein detected in each sample was determine based on the densities obtained with Image Lab Version 4.0.1 (Bio-Rad).
3.3.3.3 Re-probing membrane for GAPDH

Cell lysate PVDF membranes were washed with TBS/0.1% Tween-20 wash to remove the Immobilon Western Chemiluminecent HRP substrate (Millipore) prior to stripping. The membrane was then incubated with Pierce Restore™ Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes at room temperature and washed once again before blocking the membrane for an hour at room temperature as previously mentioned above. Sufficient removal of antibodies was tested with Immobilon Western Chemiluminecent HRP substrate (Millipore) before re-probing the membrane with mouse anti-human glyceraldehydes 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Millipore, 0.067 μg/mL) at 4°C overnight. Following five 3 minute TBS/0.1% Tween-20 washes, membranes were incubated with goat anti-mouse IgG-HRP polyclonal antibody (DAKO, 0.013 μg/mL) at room temperature for 1 hour, washed, visualised, exposed for 30 secs and quantified as above.

3.3.3.4 Coomassie staining for supernatant samples

After protein transfer of supernatant-loaded samples, gels were washed with distilled water for 10 mins at room temperature twice. Coomassie stain was then added to the gel to stain overnight at room temperature, covered until staining has been achieved. Destaining was achieved with distilled water and gels were washed until sufficient destaining was achieved.

3.3.3.5 Analysis

The densities of fibulin-1 bands for each membrane were normalised according to the normalising factor of each membrane. For each membrane, the normalisation factor was calculated as the ratio of the 100kDa fibulin-1 band density detected in the normalising healthy plasma sample on the membrane against a representative membrane. Cellular fibulin-1 was then normalised to GAPDH whilst secreted fibulin-1 was normalised to the 70kDa band in the Coomassie Blue-stained gel of each corresponding sample, and results were expressed as a fold change.
3.3.4 ECM ELISA

*In vitro* deposition of fibulin-1 on fibronectin-coated polystyrene 96-well plates was measured by ELISA according to the method previously described (229). Fibronectin was used to coat 96-well plates as it has been shown that fibronectin binds to fibulin-1 and its assembly is required to allow fibulin-1 to be incorporated into the basement membrane matrix (161). Briefly, 96-well plates were coated with 500 ng fibronectin/well at 37°C for 2 hours and washed with PBS three times before cells were seeded described above. 10% FBS was added to each plate as a positive, normalising control. Cells were lysed with 0.016 mM NH₄OH at 37°C for 20 minutes. The cell free plate ECM plates were washed with PBS three times and stored with 100 μL PBS/well at -20°C until analysis. Experiments were conducted thrice in triplicates.

ECM plates were defrosted at room temperature; PBS was removed and blocked with 1% BSA (Sigma Aldrich) in PBS at room temperature for 1 hour. ECM plates were incubated at room temperature for 2 hours with either 50 μL/well of mouse anti-human fibulin-1 monoclonal antibody (0.8 μg/mL) (Santa Cruz) as the primary antibody and mouse IgG2a antibody (0.8 μg/mL) (abcam) as the isotype control. After two PBS/0.5% Tween-20 washes, polyclonal goat anti-mouse IgG-HRP antibody (1.5 μg/mL) (Dako) was incubated at room temperature for 1 hour. All antibodies were diluted in 1% BSA/PBS/0.5% Tween-20. Plates were then washed with PBS/0.5% Tween-20 twice before 50 μL of chromogenic substrate 3, 3′, 5, 5′-tetramethylbenzidine was added and incubated at room temperature (covered) for 30 minutes. The reaction was stopped with 25 μL 1 M H₂SO₄ and absorbance was read at 450 nm and 570 nm using a FLUOstar Omega spectrophotometer (BMG Labtech). Raw values were corrected by calculating the difference in absorbance at 540 nm from 450 nm.
was conducted across the ECM plates according to 10% FBS on each ECM plate, and fibulin-1 expression levels were adjusted to the normalised values of isotype control for each cell line.

### 3.3.5 CXCL1 ELISA

Soluble and cellular CXCL1 were measured using human CXCL1 DuoSet ELISA kits (R&D systems) according to manufacturer’s protocol. Briefly, plates were coated with 4 µg/mL capture antibody at room temperature overnight. Following three washes with wash buffer (R&D), plates were blocked with reagent diluent (R&D) for 1 hour at room temperature. After washing, 100 µL of diluted sample (1:10 for supernatant and 1:5 for cell lysate) and a seven-point standard curve (0 – 2000 pg/mL) were incubated in duplicates for 2 hours at room temperature. Then 40 ng/mL detection antibody was incubated for 2 hours at room temperature following washing. Plates were washed before streptavidin-HRP was added and incubated for 20 minutes at room temperature, and washed again. Substrate solution (1:1 colour reagent A (hydrogen peroxide) and colour reagent B (tetramethylbenzidine)), 100 µL, was incubated for 20 minutes at room temperature before the reaction was terminated with 50 µL 1 M H₂SO₄. Absorbance was read within 30 minutes at 450 nm and 570 nm using a FLUOstar Omega spectrophotometer (BMG Labtech). Raw values were corrected by calculating the difference in absorbance at 540 nm from 450 nm, and concentrations were calculated using a four parametric logistic fit of the standard curve (r² > 0.99) with MyAssays (http://www.myassays.com/quantikine.assay, R&D).

### 3.3.6 RNA extraction, determination and transcription

Prior to RNA extraction, cells were removed of RNA later® by washing with equal amounts of ice-cold PBS. Total RNA was extracted with PureLink RNA Mini Kit (Life Technologies) according to manufacturer’s protocol. Briefly, cells were lysed with lysis buffer and homogenised by passing the lysate through an 18-gauge needle 10 times. The homogenate was centrifuged at 12,000g in a series of ethanol and wash buffer washes at room temperature. Purified RNA was
then eluted with 50 µL of RNase-free water. RNA concentration and quality were analysed using the NanoDrop (Thermo Scientific) and stored at -20°C until required for RNA transcription. RNA purity was assessed by examining the ratio of absorbance at 260 and 280 nm ($A_{260/280}$); a $A_{260/280} > 2$ indicated samples had high RNA purity.

RNA (2 µg) was reversed transcribed into cDNA in a reaction volume of 20 µL with SuperScript III First-Strand Synthesis SuperMix (Life Technologies) according to manufacturer’s protocol in a Bio-Rad T100 Thermal Cycler (Bio-Rad). Briefly, samples were incubated with 2.5 µM oligo(dT)$_{20}$ primers to anneal at 65°C for 5 minutes and then cooled on ice for a minute. Amplification of RNA was conducted at 50°C for 50 minutes with SuperScript III reverse transcriptase, 5 mM MgCl$_2$ and 500 µM each dNTP and the reaction was terminated at 85°C for 5 minutes. The cDNA was stored at -20°C until analysis with quantitative real-time PCR.

### 3.3.7 Quantitative real-time PCR

#### 3.3.7.1 Optimisation of primer conditions

A literature and online primer library search was conducted for Quantitative real-time PCR (qRT-PCR) primers targeting fibulin-1, CXCL1, CXCR2, MMP-13 and housekeeper, 60S ribosomal protein L32 (RPL32) resulting in the list of primers to test and optimise (Table 11). The criteria for the primer sequence were as following:

- primer sequence was restricted to 25bp
- aim to yield an amplicon length of around 150 – 400bp
- Tm (melting temperature) of primers were around 50-60°C
- C + G content was around 40 – 60%
- primer sequence crossed over exon-exon boundaries over a large intron length
- a BLAST search of the primer sequence revealed the gene and organism of interest match up; ideally would want a query coverage close to 100% and E-value of < 0.01.
Table 11. List of primer sequences to optimise for qRT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibulin-1 (1) (F)</td>
<td>TGCGAATGCAAGACGG</td>
<td>(150)</td>
</tr>
<tr>
<td>Fibulin-1 (1) (R)</td>
<td>CGTAGACGTTGCCACA</td>
<td></td>
</tr>
<tr>
<td>Fibulin-1 (2) (F)</td>
<td>TGCTTCTGGGCTACCCAGCTGCTG</td>
<td>(146)</td>
</tr>
<tr>
<td>Fibulin-1 (2) (R)</td>
<td>CTCTCTGTTGAGTAGTGAGCCACCC</td>
<td></td>
</tr>
<tr>
<td>Fibulin-1 (3) (F)</td>
<td>GGAGCAGTGCTGCCACAG</td>
<td>(153)</td>
</tr>
<tr>
<td>Fibulin-1 (3) (R)</td>
<td>AGCACCTCTTCAAAATGTG</td>
<td></td>
</tr>
<tr>
<td>RPL32 (F)</td>
<td>TTGACAACAGGGTGTGGTAGA</td>
<td>Designed in Hons project (2009)</td>
</tr>
<tr>
<td>RPL32 (R)</td>
<td>GGAAACATTGTGAGCGATCTC</td>
<td></td>
</tr>
<tr>
<td>CXCL1 (F)</td>
<td>AACCGAAGTCATAGCCACAC</td>
<td>(231)</td>
</tr>
<tr>
<td>CXCL1 (R)</td>
<td>GAGGATTGGTACTGTGTCA</td>
<td></td>
</tr>
<tr>
<td>CXCR2 (F)</td>
<td>AACATGGAGAGTGACAGCTTG</td>
<td></td>
</tr>
<tr>
<td>CXCR2 (R)</td>
<td>TCACTGGGCGGCACTC</td>
<td></td>
</tr>
<tr>
<td>Fibulin-1 (4) (F)</td>
<td>TGCGAATGCAAGACGGTTA</td>
<td>Primer bank ID: 154091330c3</td>
</tr>
<tr>
<td>Fibulin-1 (4) (R)</td>
<td>CGTAGACGTTGCCACCTC</td>
<td></td>
</tr>
<tr>
<td>MMP-13 (F)</td>
<td>CAGACTTCTACGATGGCCAATG</td>
<td>Primer bank ID: 296010793c3</td>
</tr>
<tr>
<td>MMP-13 (R)</td>
<td>GGCTCTTCCCATAATTTGGC</td>
<td></td>
</tr>
</tbody>
</table>

Optimisation of the qRT-PCR protocol for each primer was based on the protocol listed below, the annealing and elongation temperature for the cycles was altered from 60 – 70°C, to determine the optimal temperature required for maximum amplification of PCR products with high reaction efficiency and the production of a single, specific product.

For RPL32 (Figure 25), fibulin-1 (Figure 26), CXCL1 (Figure 27), CXCR2 (Figure 28) and MMP-13 (Figure 29), amplification of PCR products for these genes occurred between cycle 15 – 40. The quantitative amplification plots (log scale) were threshold at the exponential phase to indicate the point where the fluorescence is in a linear relationship (A of each figure). The standard curves of log concentration against Ct values have good correlation coefficients and optimal PCR efficiency (B of each figure) and the melt curves for each gene displayed a single peak for each sample and no peak in the negative control, thus confirming the amplified PCR products were specific and ensures there were no primer dimers in the reaction (C of each figure).
Figure 25. Optimised qRT-PCR protocol for house-keeper gene RPL32. Quantitative amplification plots (log scale) show good amplification of RPL32 (A) at optimal efficiency (B) and the melt curves confirms amplified PCR products were specific and no primer dimers were present (C).
Figure 26. Optimised qRT-PCR protocol for target gene fibulin-1. Quantitative amplification plots (log scale) show good amplification of fibulin-1 (A) at optimal efficiency (B) and the melt curves confirms amplified PCR products were specific and no primer dimers were present (C).
Figure 27. Optimised qRT-PCR protocol for target gene CXCL1. Quantitative amplification plots (log scale) show good amplification of CXCL1 (A) at optimal efficiency (B) and the melt curves confirms amplified PCR products were specific and no primer dimers were present (C).
Figure 28. Optimised qRT-PCR protocol for target gene CXCR2. Quantitative amplification plots (log scale) show good amplification of CXCR2 (A) at optimal efficiency (B) and the melt curves confirm amplified PCR products were specific and no primer dimers were present (C).
Figure 29. Optimised qRT-PCR protocol for target gene MMP-13.
Quantitative amplification plots (log scale) show good amplification of MMP-13 (A) at optimal efficiency (B) and the melt curves confirms amplified PCR products were specific and no primer dimers were present (C).
3.3.7.2 qRT-PCT protocol

Expression of fibulin-1, CXCL1, CXCR2 and MMP-13 mRNA levels was performed by a Rotor Gene 6000 system (Corbett Life Science) using SYBR Select Master Mix (Life Technologies). The SYBR Select Master Mix contains SYBR GreenER™ dye, which detects double-stranded DNA products during qRT-PCR; DNA polymerase; uracil-DNA glycosylase and dNTPs. A standard sample was containing 2 μL of cDNA from all CRC samples and diluted 1:1 with TE buffer was constructed; from this standard sample, a five-point standard curve was generated with a serial dilution of 1:4. Standard and diluted cDNA sample (1:50), 5 μL, was used in a 20 μL final reaction volume; RNase-free water was used as a negative control. The cycling conditions consist of an incubation at 50°C for 2 minutes to activate uracil-DNA glycosylase, followed by 95°C for 2 minutes to activate DNA polymerase and 50 cycles of 95°C for 15 seconds to denature and 60°C for 45 seconds to anneal and extend the cDNA template; fluorescence data was acquired at the end of each cycle. A melt curve was obtained for each gene starting at 60°C and ending at 95°C, with a 1°C increment after each 5 seconds to ensure the amplified product was specific and no primer dimers were present. Primers optimised for fibulin-1, CXCL1, CXCR2 and RPL32 are listed in Table 12.

Table 12. Optimised primer sequence used for qRT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL32 (F)</td>
<td>TTGACAACAGGGTCTCGAGA</td>
</tr>
<tr>
<td>RPL32 (R)</td>
<td>GGAAACATTGTGACGATCTC</td>
</tr>
<tr>
<td>Fibulin-1 (F)</td>
<td>TGCGAATGCAAGAGCGGTGA</td>
</tr>
<tr>
<td>Fibulin-1 (R)</td>
<td>CCGTAGACGGTGCACACTC</td>
</tr>
<tr>
<td>CXCL1 (F)</td>
<td>AACCAGAGTTGACTGACCGACAC</td>
</tr>
<tr>
<td>CXCL1 (R)</td>
<td>GTTTGAATTTGTACTGTTCAAGC</td>
</tr>
<tr>
<td>CXCR2(F)</td>
<td>AACATGGAGAGTGCACGCTTTG</td>
</tr>
<tr>
<td>CXCR2 (R)</td>
<td>TTCACATGGGCGCGCATC</td>
</tr>
</tbody>
</table>

The threshold cycle (CT) value was calculated automatically from the Rotor-Gene 6000 Series software (Corbett) and plotted against the logarithmic concentration of the standard curves.

Relative quantitative evaluation for each target gene was expressed as $2^{\Delta CT}$, where $\Delta CT$ for
each sample is represented as CT (sample) – CT (sample with max CT for the target gene), and normalised to house keeper gene, ribosomal protein L32 ($RPL32$).

$$\text{Relative mRNA expression} = \frac{2^{\Delta CT \text{ gene}}}{2^{\Delta CT \text{ RPL32}}}$$

### 3.3.8 Wound scratch assay

To examine the functional role of fibulin-1 on migration, I used two high (Caco-2 and SW620) and two low (HT29 and SW48) secreted fibulin-1-expressing CRC cell lines to conduct wound scratch assays; as CCD-18Co fibroblast was found to highly express secreted fibulin-1, I used this fibroblast cell line to compare the migration rates of the CRC cells as well. CCD-18Co fibroblast, Caco-2, SW620, SW48 and HT29 were seeded in 12 replicates overnight in a 96-well plate to form a confluent monolayer according to the seeding densities listed in Table 10.

Wounds were created with the 96-well WoundMaker (Essen Bioscience), washed twice with PBS to remove cellular debris before cells were treated with or without 10 ng/mL IL-6 in quiescing and complete media for 96 hours.

Two still images (10X objective) were acquired from the centre of each well every 2 hours for the entire duration of the assay with a real-time cell imaging system, IncuCyte™ ZOOM (Essen Bioscience), in which cells are imaged inside an incubator under optimal physiological conditions for the entire duration of the assay. The assay was quantified by measuring the wound width at each time point. For each treatment condition for each cell, samples were evaluated in six replicates from three independent experiments conducted in quiescing and complete media.

The amount of wound closure was expressed as:

$$\text{Wound closure (at t = x)}(\%) = \left( \frac{(\text{wound width at } t = 0) - (\text{wound width at } t = \text{x})}{\text{wound width at } t = 0} \right) \times 100$$
And the rate of closure was calculated as:

\[
\text{Rate of closure (μm/hr)} = \frac{\text{(wound width at } t = 0) - \text{ (wound width at closure)}}{\text{duration for wound to close}}
\]

3.3.9 HUVEC tube formation assay

To examine the functional role of fibulin-1 on angiogenesis, HUVECs were grown on a growth factor reduced basement membrane matrix with CM from CRC cells. HUVECs were grown in a T75 cm\(^2\) flask until they reached about 80% confluent. After thawing Geltrex\textsuperscript{®} LDEV-free reduced growth factor basement membrane matrix (Life Technologies) overnight at 4°C, a 96-well tissue culture plate was coated with 50 μL Geltrex\textsuperscript{®} and set aside at 37°C for 30 minutes to allow it to polymerise prior to seeding of HUVECs. After a gentle trypsin digest, HUVECs were resuspended in Media 200PRF (positive control), DMEM (0.1% FBS), DMEM (10% FBS) (both DMEM conditions served as respective CM-controls for serum content) and CM from CRC at a concentration of \(1.5 \times 10^5\) cells/mL and gently seeded \((1.5 \times 10^4\) cells/well) in triplicates in the wells coated with Geltrex\textsuperscript{®}. The plate was then incubated at 37°C and tube formation was monitored for 24 hours in the IncuCyte™ ZOOM (Essen Biosciences). Four still images (10X objective) were acquired every hour from each well. Independent experiments were conducted twice in duplicates. To measure the extent of tube formation for each condition, number of junctions, branches and meshes, and total master segment length were quantified by the plug-in, Angiogenesis Analyzer, developed for ImageJ software (NIH) (written by Gilles Carpentier (232) and is available online: (http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis\%20Analyzer.txt).

3.3.9.1 Acquisition and processing of HUVEC tube formation data

Using complete media conditions, the angiogenesis assay was initially ran to identify the optimal time period to capture the angiogenic process. I observed that within 2 hours post-seeding, HUVECs underwent rapid realignment and migrated towards each other to begin the tube formation process. By 6 hours, tubes and networks were subsequently established and
completed by 14 hours before HUVECs undergo apoptosis after 24 hours. Therefore, tube formation analysis was conducted on images taken 14 hours post-seeding.

Prior to analysis with the ImageJ (NIH) plug-in, Angiogenesis Analyzer, images acquired by the Incucyte™ ZOOM (Essen Biosciences) were batched processed in Photoshop (Adobe) by applying a Gaussian blur filter (radius = 1) as this removed the cellular debris from the background and provides more accurate analysis of the tube formation; the debris would generate artificial network elements, and thus overestimate the tube formation parameters (Figure 30A). Processed phase-contrast images were then batched process and analysed with Angiogenesis Analyzer on Image J (NIH), generating a segmented and skeletonised image revealing the various elements of the tube formation process to be analysed (Figure 30B). The elements that make up the tube network can be easily visualised by obtaining a map of the skeleton network which shows the branches (elements arising from a junction and ending at an extremity), master segments (sections of the network delimited by two master junction points), master junction points (junctions which link at least three master segments) and meshes (areas enclosed by segments) (Figure 30C).
Figure 30. Acquisition of tube formation parameters for analysis. A representative image of HUVECs cultured with CM from SW48 (10% FBS) at 10X magnification, scale bar represents 300μm (A) was processed with Photoshop (Adobe) and a Gaussian blur filter (radius = 1) was applied to obtain accurate parameters for analysis with Angiogenesis Analyzer (B). A generated map of the tube network allows for easy visualisation of master junctions (dots surrounded by outer circle), branches (green lines), master segments (yellow line) and meshes (blue enclosed lines) (C).
Chapter 3 In vitro investigation of fibulin-1 expression and functional role in colorectal cancer

3.3.10 Statistical analysis

For each cell line, three samples were obtained from three individual experiments. For the analysis of mRNA and protein expression levels, these experiments were conducted thrice and the mean was calculated across the three experiments. Data analysis was performed with SPSS Version 21.0 (IBM). All the data is represented as mean ± error (SEM), unless stated otherwise.

Comparison of fibulin-1, CXCL1, CXCR2 and MMP-13 expression, and rate of wound closure, with or without IL-6 treatment, was performed using the paired Student’s t-test. Non-parametric Mann Whitney U-test was conducted to examine the relationship between the expression of secreted fibulin-1 with cellular fibulin-1 processing. P-values < 0.05 were considered significant.
3.4 Results

3.4.1 Basal expression of fibulin-1, CXCL1, CXCR2 and MMP-13 in colon fibroblast and CRC cell lines

3.4.1.1 Differential expression of fibulin-1 mRNA and three protein forms of fibulin-1

CRC cell lines SW620, Caco-2 and HCT116 had the highest fibulin-1 mRNA expression in comparison to the other four CRC cell lines and CCD-18Co fibroblast cell line, which all had low mRNA expression of fibulin-1 (Figure 31).

![Figure 31. Relative mRNA fibulin-1 expression in CCD-18Co fibroblast and CRC cell lines. Three CRC cell lines, SW620, Caco-2 and HCT16, had the highest mRNA fibulin-1 expression.](image)

Using Western blotting, four bands immunogenic to fibulin-1 protein were detected in cell lysates and supernatant samples; the mature polypeptide (molecular mass of 100kDa) and three smaller fragments (molecular mass of 70, 50 and 30kDa). In CRC cells, cell lysates had detectable levels of the 100, 50 and 30kDa fragment whilst in the supernatant samples only the 100 and 70kDa fragments were detected. CCD-18Co fibroblasts only express the 100kDa fibulin-1 fragment in cell lysate and supernatant samples (Figure 32).
Figure 32. Basal expression of cellular and secreted fibulin-1 detected by Western blotting in CCD-18Co fibroblast and six CRC cell lines in a representative blot. CRC cells have differential expression of cellular fibulin-1 (A) whilst only SW620, Caco-2 and HCT116 have the highest expression of secreted fibulin-1 (B). CCD-18Co only express the 100kDa fibulin-1 fragment for both cellular (C) and secreted (D) forms. Abbreviations: FN indicates fibronectin (the negative control), N ctrl is the normalising healthy plasma sample control, Co. B is Coomassie Blue staining.
Western blotting analysis of basal cellular and secreted fibulin-1 in three independent experiments revealed varying levels of fibulin-1 expression across CCD-18Co fibroblasts and the six CRC cell lines. CCD-18Co fibroblasts and Caco-2 had the highest expression of the cellular 100kDa complete fibulin-1 polypeptide fragment in comparison to the other CRC cell lines. An increase in the expression of the two smaller cellular fragments (50 and 30kDa) was observed in all CRC cell lines except in Caco-2 cells (Figure 33A). However, there were no significant differences in the total cellular fibulin-1 (the sum of all three fibulin-1 fragments) observed (Figure 33C). Only CCD-18Co fibroblasts and three CRC cell lines, SW620, Caco-2, and HCT116, had high expression of secreted 100kDa fibulin-1 fragment. The expression of the secreted 70kDa fibulin-1 fragment was also observed in SW620, Caco-2, and HCT116 and at a lower level by SW48 (Figure 33B). The other remaining CRC cell lines exhibited no secreted fibulin-1 expression. When the expression of the 100kDa and 70kDa fragments was combined, CCD-18Co fibroblasts, SW620, Caco-2 and HCT116 had the highest expression of total secreted fibulin-1 (Figure 33D). All cell lines deposited fibulin-1 protein into the fibronectin-coated plates. ECM ELISA analysis of fibulin-1 deposited into the ECM also showed no differential basal expression amongst CCD-18Co fibroblasts and all CRC cell lines (Figure 33E). Due to the low numbers of cell lines that expressed fibulin-1, I could not statistically determine the relationships between mRNA and protein expression levels. I found cell lines which expressed high fibulin-1 mRNA had high levels of secreted fibulin-1 expression; with the exception of CCD-18Co fibroblasts. Also, there were no associations between the three protein forms of fibulin-1 observed.

It is still unknown of the origin of the smaller cellular fibulin-1 fragments (30 and 50kDa) detected by Western blotting. Greene et al suggested that the smaller fibulin-1 fragments are generated by proteolysis fibulin-1 polypeptide (100kDa) into smaller fragments in breast cancer and used the ratio of the 50kDa fragment to the 100kDa fragment as an indicator of fibulin-1 proteolysis (143). In my study, I detected two smaller fragments (50 and 30kDa) in the
Western blotting experiments and calculated the ratio between smaller fragments and the mature polypeptide to determine whether cellular processing of fibulin-1 affects secreted fibulin-1 levels. I found a significant inverse relationship between the proteolysis of cellular fibulin-1 and levels of total secreted fibulin-1 (p = 0.02) (Figure 33F).

Figure 33. Basal expression of cellular and secreted fibulin-1 detected by Western blotting and deposited fibulin-1 detected with ELISA in CCD-18Co fibroblast and six CRC cell lines. No differential expression of cellular fibulin-1 could be observed amongst the cell lines (A & C); however CCD-18Co, SW620, Caco-2 and HCT116 have the highest expression of secreted fibulin-1 (B & D). Deposited fibulin-1 revealed no differential expression amongst all cell lines (E). High proteolysis of cellular fibulin-1 was significantly correlated with no-to-low levels of secreted fibulin-1 (F).
3.4.1.2 Expression of \textit{CXCL1} and \textit{CXCR2} mRNA, and cellular and secreted protein CXCL1

When analysing mRNA expression of \textit{CXCL1} in the cell lines, I observed that cell lines with high \textit{fibulin-1} mRNA expression (SW620, Caco-2 and HCT116) had low mRNA expression of \textit{CXCL1}; and vice versa, cell lines with low \textit{fibulin-1} mRNA expression had high \textit{CXCL1} mRNA expression, with the exception of HT29 and SW48 (Figure 34A). The mRNA expression of \textit{CXCR2} was variable amongst all cell lines and did not appear to have any relationship with \textit{CXCL1} and \textit{fibulin-1} gene expression (Figure 34B).

![Figure 34. Relative mRNA expression of \textit{CXCL1} and \textit{CXCR2} in CCD-18Co fibroblasts and CRC cell lines. CRC cell line LIM2405 had the highest relative mRNA \textit{CXCL1} expression (A) and \textit{CXCR2} mRNA expression for CCD-18Co and the CRC cell lines were varied (B).]
As anticipated, the CRC cell lines with low CXCL1 mRNA expression (SW620, Caco-2 and HCT116) had low basal expression of cellular and secreted CXCL1 protein; and vice versa, CRC cells with high CXCL1 gene expression had high basal expression of cellular CXCL1 protein (with the exception of HT29 and SW48) (Figure 35). Levels of cellular and secreted CXCL1 were observed to be associated with relative CXCL1 mRNA expression and each other.

3.4.1.3 Expression of MMP-13 mRNA levels

As anticipated, cell lines with low fibulin-1 mRNA expression (HT29, LIM2405, and SW48) had higher mRNA expression of MMP-13, with the exception of LIM2405 and SW48; and vice versa, cells lines with high fibulin-1 mRNA expression had reduced MMP-13 mRNA expression (Figure 36). Due to time constraints, cellular and secreted protein expression of MMP-13 was not assessed in this research project to determine whether there was increased fragmentation of the secreted fibulin-1.
3.4.2 Inflammation-regulated expression in CRC and fibroblast cell lines

3.4.2.1 Fibulin-1 expression does not differ in the presence of IL-6

Treating CCD-18Co fibroblasts and the CRC cells with IL-6 for 72 hours did not significantly alter fibulin-1 mRNA expression (Figure 37) and expression of cellular (Figure 38), secreted (Figure 39) and deposited fibulin-1 protein (Figure 40).

Figure 36. Relative mRNA expression of MMP-13 in CCD-18Co fibroblasts and CRC cell lines. Differential mRNA expression of MMP-13 was observed in these cell lines.

Figure 37. Relative mRNA expression of fibulin-1 in CCD-18Co fibroblasts and CRC cell lines with the presence of IL-6 as an inflammatory stimulus. Treating the cells with IL-6 did not alter fibulin-1 mRNA expression.
Figure 38. Expression of cellular fibulin-1 detected by Western blotting in CCD-18Co fibroblast and six CRC cell lines with and without IL-6 treatment.

No significant differences in cellular fibulin-1 expression were observed in CRC (A) and CCD-18Co cell lines (B) with IL-6 treatment. Total cellular fibulin-1 remained fairly similar across all cell lines (C) with IL-6 treatment, except for SW48 and SW620. Abbreviations: N = healthy plasma normalising control; FN = fibronectin.
Figure 39. Expression of secreted fibulin-1 detected by Western blotting in CCD-18Co fibroblast and six CRC cell lines with and without IL-6 treatment. No significant differences in secreted fibulin-1 expression were observed in CRC (A) and CCD-18Co cell lines (B) with IL-6 treatment. Total secreted fibulin-1 remained fairly similar across all cell lines (C) with IL-6 treatment. Abbreviations: N = healthy plasma normalising control; FN = fibronectin; Co.B = Coomassie Blue staining.
3.4.2.2 Expression of CXCL1 and CXCR2 in the presence of IL-6

IL-6 treatment did not significantly alter the mRNA expression of CXCL1 and CXCR2 (Figure 41); and cellular or secreted CXCL1 protein (Figure 42), with the exception of SW620 which had decreased expression of secreted CXCL1 with exposure to IL-6 (p = 0.017).
Figure 42. Expression of cellular and secreted CXCL1 with and without IL-6 treatment. IL-6 treatment has no effect on cellular (A) and secreted (B) CXCL1 expression, except for secreted SW620 which is reduced with IL-6 treatment. * indicates p-value < 0.05.

3.4.2.3 Expression of MMP-13 in the presence of IL-6

Treating the cells with IL-6 did not significantly alter the mRNA expression of MMP-13, with the exception of Caco-2 which increased MMP-13 mRNA expression (P = 0.0018) (Figure 43).

Figure 43. Relative mRNA expression of MMP-13 in CCD-18Co fibroblast and CRC cell lines with the presence of IL-6 as an inflammatory stimulus. With IL-6 treatment, there was no change to mRNA expression MMP-13, with the exception of Caco-2 where MMP-13 is significantly increased. ** indicates p-value < 0.01.
3.4.3 Functional role of fibulin-1

Due to time constraints and stronger evidence that fibulin-1 regulates migration and angiogenesis; I focused on these two functional outputs to assess the effect of fibulin-1 in CRC carcinogenesis. I did not look into proliferation and invasion functional assays as current literature suggest fibulin-1 plays an indirect role in promoting these properties through CXCL1, whereas fibulin-1 interacts with fibronectin to regulate migration, and either angiogenin or endostatin to regulate angiogenesis (154, 161, 164, 171).

3.4.4.1 Migration

Representative images of CCD-18Co fibroblasts (Figure 44), Caco-2 (Figure 45), SW620 (Figure 46), SW48 (Figure 47) and HT29 (Figure 48) at 0, 24, 48, 72 and 96 hours post-wound scratching without IL-6 treatment. Over a 96 hour period, CCD-18Co fibroblast cells showed a faster migration rate (i.e. wound closure) than the four CRC cell lines in both quiescing and complete media (Figure 49A). In quiescing media (0.1% FBS containing media), Caco-2 had the highest rate of wound closure compared to the other CRC cell lines, followed by HT29; SW48 and SW620 were similar and had the lowest migration rates. Migration rates were enhanced with complete media (media + 10% FBS) in comparison to results of cells in quiescing media. The migration rate for Caco-2 in complete media was significantly increased and was faster than that of CCD-18Co fibroblasts, whilst the migration rate of SW620 and SW48 in complete media increased to match the same migration rate of HT29, which did not change with the addition of complete media (Figure 49B).

Supporting the fibulin-1 protein and mRNA expression experiments conducted under inflammatory conditions above, treating the cells with IL-6 had no effect on migration rates and wound closure of CRC cell lines (Figure 50).
Figure 44. Representative images of wound scratch assay over a 96 hour time-course for CCD-18Co fibroblast without IL-6 treatment; 10X magnification, scale bar represents 300μm.
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Figure 45. Representative images of wound scratch assay over a 96 hour time-course for Caco-2 without IL-6 treatment; 10X magnification, scale bar represents 300μm.
Figure 46. Representative images of wound scratch assay over a 96 hour time-course for SW620 without IL-6 treatment; 10X magnification, scale bar represents 300 μm.
Figure 47. Representative images of wound scratch assay over a 96 hour time-course for SW48 without IL-6 treatment; 10X magnification, scale bar represents 300μm.
Figure 48. Representative images of wound scratch assay over a 96 hour time-course for HT29 without IL-6 treatment; 10X magnification, scale bar represents 300μm.
Figure 49. The extent of wound closure in wound scratch assays of CRC cell lines over 96 hours with 0.1% (quiescing) and 10% (complete) FBS. Wound closure (%) (A) and rate of closure (B) over a 96hr period shows that CCD-18Co and Caco-2 cell lines have the fastest migration ability compared to the other CRC cell lines.
Figure 50. The extent of wound closure in wound scratch assays of CRC cell lines over 96 hours with 0.1% (quiescing) and 10% (complete) FBS, with and without IL-6 treatment.
IL-6 treatment does not affect wound closure (%) (A) or the rate of closure (B).
3.4.4.2 Angiogenesis

Secreted fibulin-1 protein has been shown to regulate angiogenesis in renal cancer and fibrosarcoma (151, 179). In this experiment I aimed to elucidate whether secreted fibulin-1 derived from CRC cell lines promotes angiogenesis. I utilised the quiescing and complete CM from 4 CRC cell lines; two which are high-fibulin-1 secreting and two which are low-fibulin-1 secreting, as it has been shown that HUVECs do not express fibulin-1 (139).

In the presence of 0.1% FBS, CM from three of the CRC cell lines (Caco-2, HT29 and SW620), showed enhanced tube formation compared to the negative control. With complete CM (containing 10% FBS) tube formation was similar amongst all CRC cells and the CM-control and positive control (Media200 PRF) (Figure 51)

Within the vessel network, the ‘meshes’ and ‘junctions’ components could provide an indication of the complexity of the complete tubes formed, while the ‘branches’ indicate evidence of new sprouting vessels yet incomplete tubes. The total master segment length measurement of the network calculates the total length of all the segments for the condition and would provide an indication of the extent of tube formation. CM from CRC cell lines enhanced the number of meshes, branches and junctions in comparison to the media control at low serum concentrations (0.1% FBS) (Figure 52A) with the number of these measurements being comparable to the media control at higher serum content (Figure 52B). The total length of the vessel network was increased to the same degree with the presence of CM from CRC cells in low serum conditions in comparison to the media control and was similar amongst all 4 CRC cell lines and controls at higher serum content (Figure 52C). There were no significant differences for any angiogenesis parameter between high and low expressing fibulin-1 CRC cell line at either FBS concentration.
Figure 51. Representative images of HUVEC tube formation assay on basement membrane matrix. Tube formation was determined by automated phase microscopy at 10X magnification, scale bar represents 300μm.
Figure 52. HUVEC tube formation assay analysis.
In low serum conditions (0.1% FBS), CM from CRC cell lines enhanced the number of meshes, branches and junctions in the vessel network in comparison to the media control (A). With increased serum (10% FBS) in the media, the number of meshes, branches and junctions were comparable to the media control (B). The total length of the vessel network was increased with the presence of CM from CRC cells in low serum conditions in comparison to the media control, and similar amongst all 4 CRC cell lines and controls at higher serum content (C).
3.5 Discussion

In Chapter 2, I found that a reduction in plasma fibulin-1 levels was associated with elevated systemic inflammation and poorer OS in mCRC patients. There is little information regarding the functional role secreted fibulin-1 plays in CRC carcinogenesis, its relationship with systemic inflammation and how it influences clinical patient outcomes.

In this Chapter, I found that there was differential expression of fibulin-1 mRNA and protein expression between one colonic fibroblast and six CRC cell lines. Cell lines that expressed high cellular fibulin-1 mRNA had higher secreted fibulin-1 expression. In addition, CRC cell lines with low fibulin-1 cellular gene and protein expression had higher levels of CXCL1 gene and secreted and cellular protein expression, and MMP-13 gene expression, which is consistent with the literature. Despite differences in basal fibulin-1 gene and protein expression in the CRC cell lines investigated, I was unable to identify functional differences in cellular migration or promotion of angiogenesis.

3.5.1 Expression and regulation of fibulin-1

In the CCD-18Co fibroblast and CRC cell lines, I found no consistent pattern of expression between fibulin-1 mRNA and all three protein forms of fibulin-1. However, fibulin-1 mRNA expression in the CRC cell lines used in this study was consistent with the study conducted by Roark et al, where no to low expression of fibulin-1 mRNA in CRC cell lines was found (139). CCD-18Co fibroblast and three CRC cell lines (SW620, Caco-2 and HCT116) had the highest levels of secreted fibulin-1 in comparison to the other CRC cell lines and were observed to be associated with high fibulin-1 mRNA levels; with the exception of CCD-18Co fibroblasts, which had very low quantities of RNA extracted and may be the reason why I was not able to detect fibulin-1 mRNA levels in this cell line. No associations were found between the expression of all three forms proteins of fibulin-1 which is consistent with my clinical findings in Chapter 2,
where the extent of fibulin-1 deposited into the ECM was not correlated with the levels of cellular and secreted fibulin-1.

The downregulation of fibulin-1 mRNA expression levels was observed to be associated with increased CXCL1 and MMP-13 mRNA levels for most of the cell lines. It is thought that secretion of CXCL1 by CRC cells reduces the expression of deposited fibulin-1 as it diffuses into the surrounding stroma and could be mediated by CXCR2 expression (153). However, in this study only the expression of secreted fibulin-1 was associated with both cellular and secreted CXCL1 levels and this observation does not seem to be mediated by CXCR2 mRNA expression.

Interestingly, the three CRC cell lines (SW620, Caco-2 and HCT116) which had higher levels of fibulin-1 mRNA expression did not have high expression of cellular and deposited fibulin-1 in comparison to the other cell lines but had high levels of the secreted form. A possible explanation for this finding is that there is altered cellular processing of fibulin-1 which hinders its secretion. In this study, I examined the ratio of smaller cellular fibulin-1 fragments (30 and 50kDa) against the mature polypeptide (100kDa) fragment as a measure of cellular fibulin-1 proteolysis to determine whether altered processing of cellular fibulin-1 was associated with the levels of secreted fibulin-1. Cells that have higher levels of cellular fibulin-1 processing had reduced levels of total secreted fibulin-1. Therefore, an alteration in the processing of cellular fibulin-1 may influence the amount of fibulin-1 secreted into the tumour microenvironment and hence, affect the tumour cell behaviour properties such as proliferation and migration.

3.5.2 Functional role of fibulin-1 in carcinogenesis

The functional role of fibulin-1 in carcinogenesis involves many different aspects such as cell proliferation, apoptosis, motility, invasion and angiogenesis (142, 151, 154, 155, 164, 179). To investigate the functional role of fibulin-1 in carcinogenesis in vitro, I used two high (Caco-2 and SW620) and two low (HT29 and SW48) secreted fibulin-1-expressing CRC cell line to
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examine the effect differential fibulin-1 expression has on migration and angiogenesis by conducting wound scratch and HUVEC tube formation assays respectively.

3.5.2.1 Migration

In the wound scratch assay, Caco-2 cells, which have the highest secreted fibulin-1 expression, was expected to have the slowest migration of all the CRC cells; however, it had enhanced wound closure and the fastest migration rate in comparison to the other cell lines. In contrast, SW48 cells, which has the lowest fibulin-1 expression, was expected to have the fastest migration rate out of all the CRC cells but it was one of the slowest migrating cell line. Unexpectedly, no associations were found between differential basal expression of secreted fibulin-1 and migration.

These results are in contrast to previously published studies that have observed a reduction in secreted fibulin-1 expression, either due to proteolysis by MMP-13, downregulation by CXCL1 or estradiol, lead to enhanced cell migration (142, 154, 157, 161). The discrepancy between the results of this study and the other studies could be due to various reasons. In the study conducted by Hayashido et al and Twal et al (142, 161), it was found that the suppressive effect of fibulin-1 on motility and migration was only observed in the presence of fibronectin. In the current study we conducted the experiments on plastic not fibronectin-coated plates and this may explain the low number of migrating cells that was observed. Furthermore, changes in fibulin-1 function were found only when high concentrations of fibulin-1 antibodies were used or via overexpression of CXCL1 or MMP-13 genes (154, 157). Whilst in my studies, I investigated the effect of basal secreted fibulin-1 expression of the cells. It is unclear which of the types of experiment (ie basal versus induced) is more representative of the clinical situation.
3.5.2.2 Angiogenesis

I had anticipated that the two high fibulin-1 expressing CRC cell lines would have reduced tube formation in comparison to the two low fibulin-1 expression CRC cell lines. However, in the HUVEC tube formation assay, CM from all 4 cell lines promoted tube formation to the same extent, regardless of absence or presence of serum in the media. Therefore, it seems that angiogenesis does not appear to be dependent on secreted fibulin-1 expression in these CRC cell lines.

These results are not consistent with the findings in the studies conducted by Xie et al and Xiao et al, where it was found that overexpression of fibulin-1 in HT1080 fibrosarcoma and 5637 bladder cancer cell lines significantly inhibited angiogenesis (149, 179). This may be due to the fact that the experiments conducted in this thesis were designed to investigate the effect of basal secreted fibulin-1 on angiogenesis, whilst the two studies previously published had genetically modified the expression of fibulin-1. Overexpression of fibulin-1 ensured that the functional effects would be more obvious in these two studies. As with the migration studies, it remains unclear how over-expression studies recapitulate the role of fibulin-1 in the tumour of patients with CRC.

3.5.3. Role of inflammation on fibulin-1 expression and cell migration in vitro

In this study, the role of inflammation in promoting angiogenesis by CRC cell lines was not investigated as the study conducted by Nagasaki et al. found IL-6 stimulation was more imperative in CAFs than CRC cell lines, HT29 and COLM5, in promoting angiogenesis (197). Stimulation of IL-6 greatly enhanced VEGF expression and angiogenesis in CAFs in vitro and the use of anti-human and anti-mouse IL-6 receptor antibodies to distinguish the blockade of IL-6 receptor signals from human CRC cells and mouse stromal cells revealed pronounced anti-tumour effects when IL-6 receptor signals were blocked in stromal cells in vivo (197).
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Despite the negative correlation of IL-6 and fibulin-1 expression I observed in my clinical studies, these in vitro studies found the effects of IL-6 on fibulin-1 expression and cell migration rates of the CRC cell lines were minimal. I had anticipated that treating the cells with IL-6 would reduce fibulin-1 expression to further enhance cell migration. In the presence of IL-6 treatment, MMP-13 mRNA expression was significantly increased in Caco-2 only. This increase in MMP-13 could explain why I observed a decrease in fibulin-1 mRNA expression in Caco-2 with IL-6 treatment. However, none of the CRC cell lines had enhanced migration rates with IL-6 treatment.

It is possible that the concentration of IL-6 used was insufficient to notice pronounced changes in fibulin-1 expression and cell migration. Hsu et al. found that using physiological concentrations of IL-6 (10 ng/mL) had no significant effect on cell proliferation in human CRC cell line SW480, but at pharmacological concentrations (50 ng/mL) proliferation was significantly enhanced. At this concentration, cell adhesion, invasion and chemotaxis was significantly increased; supratherapeutic IL-6 concentrations (100 ng/mL) reversed these effects, highlighting the negative feedback mechanism of IL-6 (233). Differences in receptor IL-6Rα subunit and signal transduction gp130 subunit expression in human CRC lines (234) may also explain the difference in response to IL-6 treatment observed in these in vitro experiments. For example, Caco-2 weakly expresses IL-6Rα subunit and gp-130 whilst SW620 express both these receptor subunits, due to a lack of the IL-6α subunit Caco-2 cells are unable to express a functional IL-6R complex and therefore would not be able to respond to exogenous IL-6 (234). However, since there was no difference in fibulin-1 expression and cell migrating rates for Caco-2 and SW620 with IL-6 treatment, this could suggest that either IL-6 is not essential or a plethora of cytokines are required in regulating fibulin-1.
3.5.4 Technical considerations

In this chapter, due to time constraints, I did not measure the MMP-13 protein expression levels in the cell lines nor inhibit its expression to confirm the role of MMP-13 in degrading the mature polypeptide fragment (100kDa) of fibulin-1 to elucidate any associations between the expression of protein MMP-13 and fibulin-1.

The expression and distribution of proteins from mRNA is largely governed by mRNA translation efficiency, post-translational modification, and secretion and deposition rates. However, it could be possible that the expression of ECM proteins such as fibulin-1 are more tightly controlled by the actions of extracellular proteases and are subjected to post-translational modifications in the cancer setting. However, this discrepancy between mRNA and protein expression was not observed for CXCL1 (235). Glycosylation is one of the post-translational modifications that can occur in plasma proteins which affects protein folding, degradation and secretion, and the degree of glycosylation of plasma proteins have been found to be significantly altered in cancer (236). Post-translational modifications of fibulin-1 in plasma also need to be considered to account for the discrepancies between the in vitro and clinical studies, to further explain the relationship of reduced fibulin-1 expression in CRC carcinogenesis and its influence in poorer OS observed in mCRC patients.

Basal expression of the secreted fibulin-1 in the four CRC cell lines did not appear to have a clear functional role in influencing cell migration and angiogenesis. These findings were not consistent with other studies in other cancer types. In these studies, overexpression of fibulin-1 in tumour cells was found to inhibit endothelial cell proliferation, tumour migration and invasion in vitro, reduce tumour growth, inhibit angiogenesis and promotes tumour apoptosis in vivo (142, 151, 153, 154, 179). This discrepancy between my study and others would be due to the fact that these studies utilised techniques where fibulin-1 expression either was inhibited with antibodies targeting fibulin-1 or overexpressing CXCL1, or increased by
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overexpressing fibulin-1 in malignant cells (142, 151, 153, 154, 179). These methods resulted in dramatic fold-change increases or decreases in fibulin-1 of up to 15-fold and thus functional changes were more obvious. The differences in fibulin-1 expression in these cell lines were 2 to 3-fold at most and thus this might be a possible reason I was unable to confirm previous genetically engineered in vitro studies of fibulin-1. Future studies investigating the functional role of fibulin-1 in CRC carcinogenesis need to consider completely knocking out the expression of fibulin-1 or overexpressing fibulin-1 in specific cell lines, as well as examine the effect of secreted and deposited fibulin-1 levels as a result of these forced tumour fibulin-1 expression, to conclusively elucidate the functional of all three forms of fibulin-1.

Investigating cell migration using the wound scratch assay may not have provided a good indication of the migration properties of CRC cells studied. I had not taken into consideration whether the results obtained were due to the proliferation or migration properties of the cells, as the different seeding densities used for the wound scratch assay could suggest differences in proliferation rates which would influence the migration rates I calculated in this assay. However, the seeding densities were chosen to ensure each well for the assay had reached approximately 90% confluency overnight and took into consideration that CCD-18Co and Caco-2 cells were larger in size in comparison to the other cell lines, therefore would occupy more space in the well sooner. In order to distinguish cell migration, Boyden chamber migration assays with ECM/matrigel layers, may provide a more functional output reading.

Despite the ease and affordability of conducting wound scratch assays, there are a number of disadvantage and limitations in comparison to other in vitro methods (237, 238). In these experiments, I used the WoundMaker (Essen) to create a scratch in the confluent monolayer of cells to minimise the variability of scratch sizes if there to be produced manually. I found that the scratches created in the five cell lines investigated displayed wide variability and appeared to be a function of the degree of adhesiveness seen between the cell lines. Caco-2 was the less
adherent cell line and each time the WoundMaker (Essen) had made its scratch, an area bigger than the anticipated scratch was removed off; therefore Caco-2 did not have reproducible scratches in this wound scratch assay. To compensate for this variability in scratch size, measurements of cell migration properties were calculated according to initial wound size. Although the migration rate would remain the same, it is anticipated that the time taken for the wound to close for Caco-2 would be quicker. A different approach to investigate the functional role of fibulin-1 in migration would involve conducting the migration assay in Boyden chambers with over- or under-expressing fibulin-1 in these cell lines, as well as knocking out the effect of fibulin-1 using antibodies.

3.5.5 Conclusions

The fibulin-1 expression and functional in vitro studies conducted in this chapter have not provided conclusive insights into my clinical findings of reduced plasma fibulin-1 being associated with poor OS in mCRC patients with elevated pro-inflammatory cytokines. Perhaps there are other regulatory mechanisms of secreted fibulin-1 in dictating clinical outcomes, which are dependent on the source of its production. Further investigations are required to identify the source of fibulin-1 in patients with cancer when the tumour and tumour-associated stromal expression is significantly reduced.
Chapter 4
General discussion and future directions
4.1 Overview of research findings

Patients diagnosed with mCRC are administrated with palliative 5-FU-based chemotherapy as their first-line of treatment to extend survival. However, there is no way of distinguishing which 50% of patients will respond to these chemotherapy regimens and have better survival outcomes. Therefore, the need for biomarkers to predict for chemotherapy response and patient survival outcomes is of high importance. By doing so, improvements to the treatment selection process could enhance chemotherapy response rates and improve patient survival.

ECM remodelling plays a vital role in the promotion of carcinogenesis and is indicative of highly invasive and advanced tumour phenotypes in mCRC patients, resulting in poorer survival rates in these patients. Fibulin-1 is an ECM protein and plasma glycoprotein found to be involved in numerous aspects of carcinogenesis and therefore could serve as a novel prognostic biomarker candidate.

In this research project, to evaluate the value of fibulin-1 in predicting clinical outcomes and response to chemotherapy in mCRC patients, the expression of fibulin-1 in clinical samples and cell lines were investigated with a variety of techniques, including IHC, targeted proteomic technique, SRM-MS, and complementary protein and RNA quantification assays, such as Western blotting and qRT-PCR.

In Chapter 2, I utilised IHC, SRM-MS and Western blotting techniques to examine fibulin-1 expression in tumour tissue and plasma. In tumour tissue, fibulin-1 was not detected in malignant CRC cells and a weak fibulin-1 staining in the stromal compartments was observed. There were detectable levels of fibulin-1 found in the plasma using SRM-MS and Western blots. A reduction of plasma fibulin-1 was associated with poorer OS and elevated systemic inflammation. This is the first time that plasma fibulin-1 has been associated with survival in patients with cancer. This novel finding warranted a more thorough investigation to explore the functional role of secreted fibulin-1 in CRC carcinogenesis to attain an understanding as to
why patients with reduced levels of fibulin-1 had poorer OS and elevated systemic inflammation in Chapter 3.

In Chapter 3, I aimed to examine the expression of cellular, secreted and deposited fibulin-1 and elucidate the regulation of fibulin-1 expression by IL-6, CXCL1 and MMP-13 in human colonic fibroblasts and six CRC cell lines. The functional role of fibulin-1 on migration and angiogenesis was assessed with wound scratch assay and HUVEC tube formation assay, respectively. I found differential expression of fibulin-1 in the cell lines investigated; cell lines which expressed high fibulin-1 mRNA had high levels of secreted fibulin-1 expression, and low levels of CXCL1 and MMP-13 mRNA. Unfortunately, I was unable to identify functional differences in cellular migration or promotion of angiogenesis according to differences in basal secreted fibulin-1 expression. Treatment of IL-6 in these cell lines had no effect on altering fibulin-1 expression or functional outcomes.

### 4.2 Evaluation of methodology

#### 4.2.1 Selective reaction monitoring mass spectrometry

SRM-MS is a highly sensitive, selective and reproducible technique for the quantification of targeted protein of interest and it is capable of multiplexing without excessive sample processing steps prior to analysis which minimises the probability of errors acquired with quantification. In this study, plasma samples were immunodepleted of the top-14 high abundant proteins as these proteins accounted for over 90% of the total protein mass in order to enhance the detection of lower abundance proteins (189). However, only recently depletion of high abundance plasma proteins in order to quantitate the lower abundance proteins has been suggested to be counter-productive due to the non-specific removal of the lower abundance proteins during the depletion process and the possible further loss of proteins in post-depletion sample preparation steps (215).
To increase the sensitivity of detecting and quantify the absolute concentration of fibulin-1 in plasma with SRM-MS, the use of stable isotope-labelled standard peptide analogues of the targeted fibulin-1 peptides would be added to the enzymatic digests of samples and quantified alongside the endogenous peptides, without the need to immunodeplete or fractionate the samples. Spiking un-depleted plasma samples with exogenous standard peptides has been shown to detect proteins of concentrations as low as ng/mL with high reproducibility, signal stability and sensitivity (216).

4.2.2 In vitro functional assays

Whilst basal expression of secreted fibulin-1 in the four CRC cell lines did not appear to have a clear functional role in influencing cell migration and angiogenesis, I cannot rule out that fibulin-1 does not play a role in influencing these processes during CRC carcinogenesis as I did not over-express or inhibit fibulin-1 expression by means of genetic engineering techniques or use of antibodies. In other studies which employed these techniques to explore the functional role of fibulin-1 in other cancer types, the increase or decrease in fibulin-1 expression from forced genetic expression resulted in dramatic differences in fold-change of up to 15-fold in comparison to differential basal expression I found, which at most were 2 to 3-fold; thus changes to these functional assay as a result of over-expressing or inhibiting fibulin-1 expression were more obvious (142, 151, 153, 154, 179). How relevant these approaches are to the clinical situation is unknown at the moment because I do not know the exact amounts of fibulin-1 protein in normal and tumour tissue that will enable a comparison for genetic models.

4.2.3 Challenges encountered

One major challenge in this research project was the small cohort size of mCRC patients obtained through our collaborators at the two Sydney hospital sites. I was able to obtain plasma samples from 32 mCRC patients for fibulin-1 analysis, and of these patients, only 19
formalin-fixed paraffin-embedded tumour samples were available for analysis of fibulin-1 in tissue sections. Although it was not the main focus of this study, collection of plasma and tissue samples from patients with inflammatory bowel disease and early CRC would have been ideal to compare the changes to fibulin-1 expression in other non-cancerous inflammatory conditions and with CRC progression. This would have allowed us to ensure that the reduction of fibulin-1 expression observed is specific, and a true indication of the carcinogenic processes occurring in mCRC patients, and thus, does not merely reflect changes in protein levels due to natural fluctuations.

4.3 A proposed role of fibulin-1 in CRC

As discussed in earlier Chapters, fibulin-1 is a multifunctional ECM protein and secreted glycoprotein, which has been shown to bind and interact with many ECM proteins. Fibulin-1 plays a vital role in cell proliferation, adhesion, migration, invasion and angiogenesis in various cancer types (136, 151, 153, 161, 179). In my in vitro studies, I found that cells with high CXCL1 and MMP-13 mRNA had low mRNA fibulin-1 expression and vice versa. This is in support of the study by Wen et al. where it was found that HCT-15 CRC cells transfected with siRNA to CXCL1 had increased fibulin-1 expression and reduced cell growth (153). Therefore, it is likely that fibulin-1 expression is either downregulated by CXCL1 or proteolysed by MMP-13 in mCRC; where the expression of these two genes are also found to be highly expressed in CRC patients and associated with poor prognosis (93, 158).

Increased plasma concentrations of fibulin-1 have been previously suggested to serve as a marker for renal impairment, and ECM remodelling, and complications in respiratory and cardiovascular diseases (132, 133, 185, 186). The molecular basis of fibulin-1 to promote renal impairment and to direct dynamic ECM changes in these diseases is not completely understood. It has been suggested that increasing levels of fibulin-1 is associated with increasing ECM stiffness (resistance to deformation) and enhanced thickening of the basement
membrane in these diseases, which leads to impaired organ function and eventually contributes to organ failure.

However in CRC carcinogenesis, it is possible that the reduction of fibulin-1 reduces the structural integrity of the ECM and decreases the inhibitory effects on stromal (pericytes, platelets, fibroblast) and tumour cellular adhesion. These changes promote easier access out of the tumour bed and enhancement of leaky tumour vasculature that assists malignant cells to intravasate and spread to distant sites.

This is the first time, to my knowledge, that a reduction of circulating fibulin-1 levels in mCRC patients has been associated with elevated systemic inflammation and poorer OS. The reduction of plasma fibulin-1 may arise from down-regulation by CXCL1 or proteolysis by MMP-13. However, there are many questions that still remain unanswered. In the IHC experiments, I observed no detectable expression of fibulin-1 in the malignant cells located in tumour tissue sections. There is no information regarding the underlying mechanisms involved in altered cellular fibulin-1 expression in dictating how fibulin-1 is secreted into the circulation or deposited into the ECM, nor the source of fibulin-1 during carcinogenesis is not completely known. Future studies need to be conducted to further understand the role of fibulin-1 in promoting CRC carcinogenesis.

4.4 Future directions

The clinical findings presented in this study provide the framework for future investigation of fibulin-1 as a prognostic biomarker in patients with mCRC. For fibulin-1 to be of clinical use, future studies need to assess the sensitivity and specificity of detection assays in a larger cohort of mCRC patients, as well as the expression of plasma fibulin-1 in patients with inflammatory bowel disease and earlier stages of CRC, which was beyond the scope of this study. By doing so, these studies could further support and add weight to my clinical findings.
of reduced plasma fibulin-1 levels predicting poor OS and ensures the specificity of this marker in mCRC patients.

For the routine clinical use of fibulin-1 as a prognostic biomarker, the development of a gold standard clinical laboratory assays such as ELISAs would be ideal. In this study, I did not use an ELISA to validate my SRM-MS proteomics findings, instead I validated my results with Western blotting using an antibody which its epitope corresponded to the sequence of amino acids 1-190 (the majority of the N-terminus) which only aligns with the SQETGDLDVGLQETDK peptide. Further research into developing clinical tests may require the development of an ELISA assay with antibodies raised against the most useful tryptic peptide target in my SRM-MS experiments (TGYYFDGISR).

To further establish the use of fibulin-1 as a prognostic marker in mCRC, other post-translational modifications need to taken into consideration. Glycosylation is one of the post-translational modifications that can occur in plasma proteins and the degree of glycosylated plasma proteins have been found to be significantly altered in cancer (236). Consistent with the study by Overgaard et al., these post-translational modifications of fibulin-1 in plasma could account for the discrepancies between the SRM-MS experiments and validating assays (239). In both studies, glycosylated (and other post-translational modification) forms of the fibulin-1 tryptic peptide were not considered during SRM-MS assay development, and analysis of the intact, full length fibulin-1 protein may have been interfered by post-translational modifications and binding proteins. Future work may also investigate the enormously complex glycoproteome of plasma fibulin-1 in understanding how post-translational modifications affect carcinogenesis and patient clinical response and outcomes.

The detection of plasma fibulin-1 provides an indication of the overall stage of carcinogenesis at a particular point in time. It is still unknown where the source of this detectable fibulin-1 in
plasma arises from and how it becomes secreted into the circulation. It has been implicated to possibly arise from fibroblasts as CAFs have been found to have decreased fibulin-1 expression in comparison to normal fibroblasts and have been re-educated to promote cancer progression in tumours (195, 240). Fibulin-1 has been also been found in fibrin clots and is capable of mediating platelet adhesion (140, 162). Activation of platelets has also been shown to play a crucial role in promoting carcinogenesis by guiding malignant cells into the blood stream, guarding malignant cells from being eliminated by immune cells within the circulation, stabilising the growth of vessels, and survival of malignant cells within secondary metastatic tissue (241). In this patient cohort, I found PLR was significantly positively correlated with NLR ($r = 0.46 & p = 0.073$) and negatively correlated with OS ($r = -0.55 & p = 0.0012$). Elevated levels of platelets have also been found to be associated with poor prognosis in CRC (242). These findings are promising and may provide further insights into the source of fibulin-1 and its role in promoting carcinogenesis.

Future *in vitro* co-culture studies conducted in CRC cell lines with and without fibroblast or platelets could elucidate the source of secreted fibulin-1 within the tumour microenvironment. Measurements of the cellular and secreted fibulin-1 expression prior to conducting the functional *in vitro* experiments could determine if the expression and function of fibulin-1 is altered in these co-culture experiments. Ideally experiments conducted *in vivo* would answer some of these questions. However, fibulin-1 knockout mice are embryolethal as a result of cardiac anomalies, haemorrhages, and deformed and delayed development of the kidneys, lungs and skull (243, 244). Therefore, to address this problem, conditional cell-specific knockouts of fibulin-1 need to be developed. This would allow mice to develop normally until adulthood where fibulin-1 expression can be silenced in a cell-specific manner (that is, in fibroblasts or platelets) and the functional role of carcinogenesis can then be investigated.
The interactions between malignant epithelial cells and, the surrounding cellular and soluble components within in the tumour microenvironment play a vital role in the carcinogenic process. Communication is feasible and mediated by the presence of a complex network of soluble mediators such as growth factors, cytokines, chemokines, steroidal hormones, and ECM components and proteases. In this research project, I found that a reduction in plasma fibulin-1 levels is associated with poorer patient survival and elevated systemic inflammation in patients with mCRC. Ultimately, determining the source of fibulin-1 reduction in plasma and understanding the role of fibulin-1 during CRC carcinogenesis may be useful as a prognostic biomarker. This would provide a novel approach to personalising treatment and allow for early intervention by clinicians should patients fail to respond and benefit from chemotherapy; and thereby improving a patient’s quality of life and survival outcomes.
Supporting material

Supplement Table 1. Raw data for each transition detected and quantitated with SRM-MS in the cohort of 32 mCRC patients.
References


78. Sengupta N, MacDonald TT. The role of matrix metalloproteinases in stromal/epithelial interactions in the gut2007.


