A Mechanistic Investigation of the Relationship Between Extramural Vascular Invasion (EMVI) and CpG Island Methylator Phenotype (CIMP) in Rectal Cancer

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

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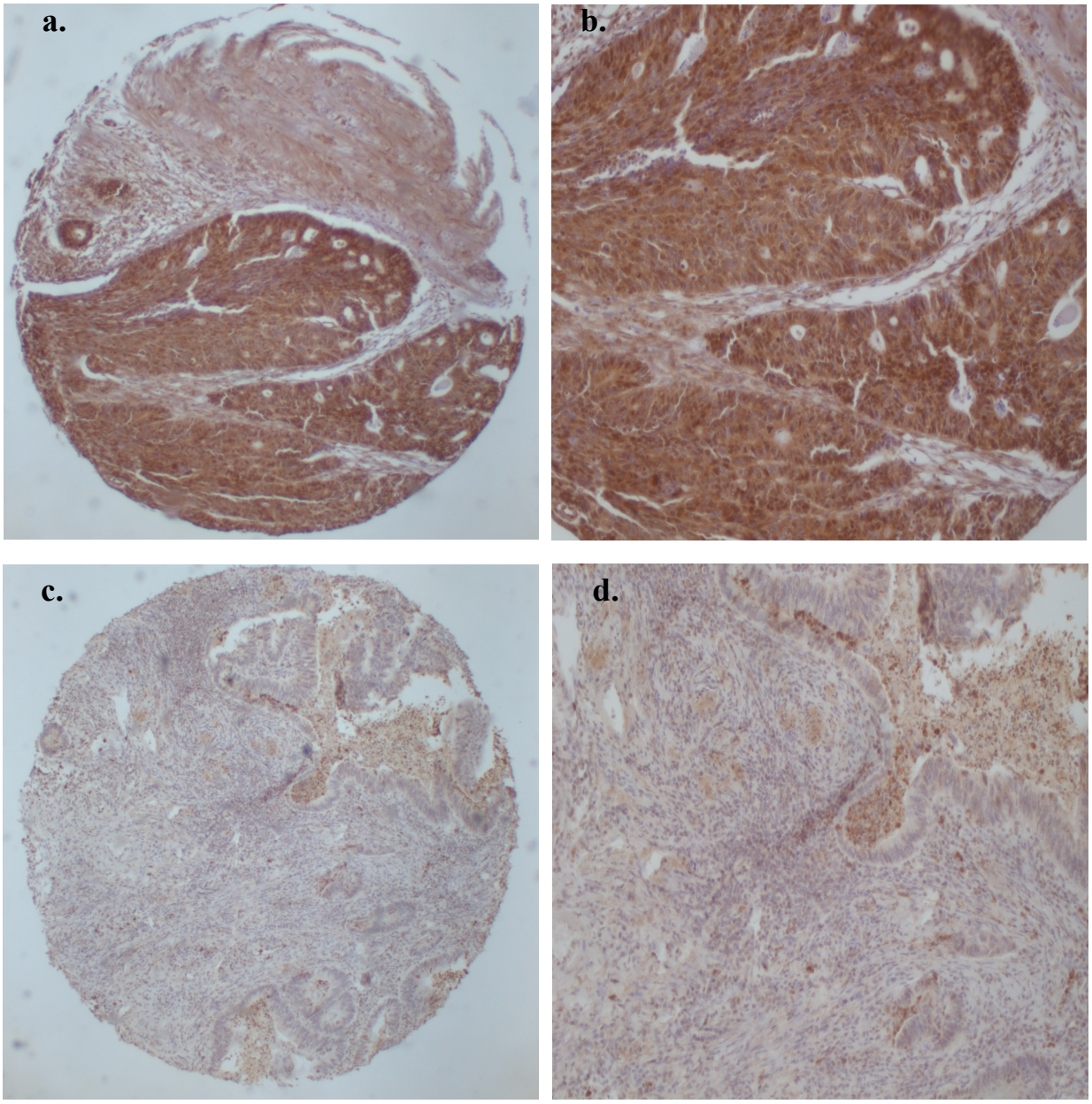
Immunohistochemistry images from an EMVI-positive tumour (a. & b.) and an EMVI-negative tumour (c. & d.) stained for metalloproteinase 2 (MMP2).



MMP2 was demonstrated to be associated with EMVI-status in resected rectal cancers (p<0.0001), was an accurate predictor of EMVI-status (Test Accuracy 0.90, AUC >0.95), and was superior to methylation status as an EMVI-status discriminator. Furthermore, MMP2 was associated with worse disease-free and overall survival in rectal cancers (p=0.030 and 0.049, respectively). Full results in **Chapter 7**.

Summary

Colorectal cancer (CRC) is the third most frequent cancer and the second leading cause of cancer death worldwide. Each year, one million people will develop CRC, and 40-50% will die within five years. Furthermore, rectal and distal sigmoid cancers are known to present at a later stage and have a poorer prognosis than other colonic cancers. Rectal cancers that demonstrate pathological extramural vascular invasion (EMVI-positive) are known to have a poorer prognosis than those that do not (EMIV-negative), and EMVI has is acknowledged as an important risk factor for systemic recurrence, local recurrence and death. Additionally, EMVI status influences the need for pre- and post-operative chemoradiation (CRT); which may influence survival outcomes.

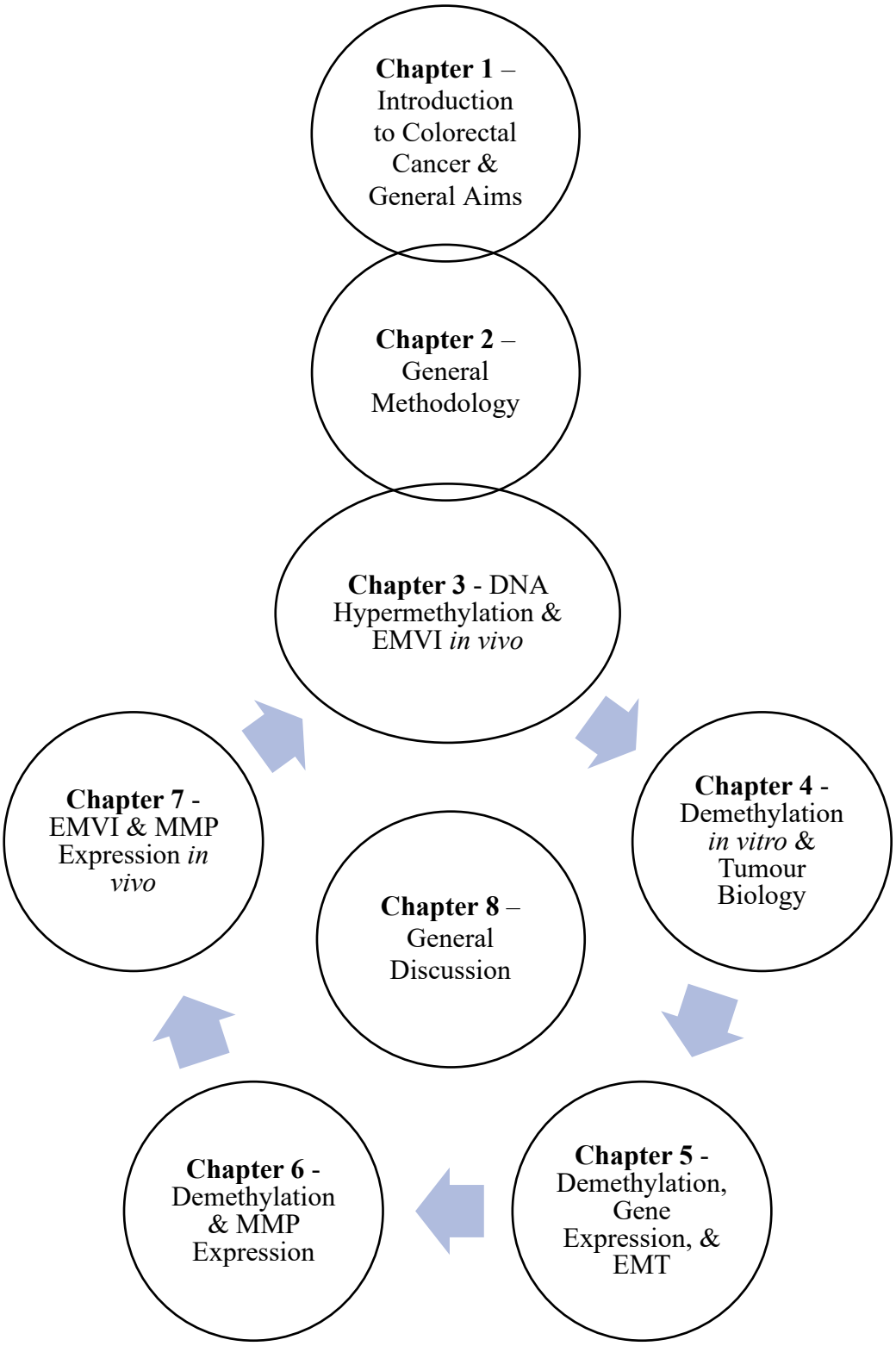
Aberrant DNA methylation is emerging as a carcinogenic mechanism and potential biomarker in colorectal cancer. This study investigates the association between hypermethylation and EMVI *in vivo* and *in vitro*. Firstly, the *in vivo* associations between hypermethylation, EMVI, and clinical and histopathological outcomes are examined. Secondly, an investigation of the effects of demethylation on invasive colorectal cell lines *in vitro* aims to illuminate the genetic and cellular mechanisms that underlie methylation-dependent pathological cellular behaviour. Finally, highlighted biologic mechanisms are investigated *in vivo* to discover if there is an association with EMVI and survival outcomes. By these means the axis of association between hypermethylation, EMVI, and clinical outcomes is investigated. The investigation is conducted within the framework of consensus molecular subtyping in colorectal cancer, and in concordance with current methodologies of assessing DNA methylation status.

The primary findings demonstrate that EMVI is associated with hypermethylation *in vivo*, but that there is no direct correlation between hypermethylation and disease-free (DFS) or overall survival (OS). *In vitro*, demethylation of hypermethylated colorectal cancer cells, by means of established demethylating agent 5-azacytidine and putative demethylator RRx-001, reduces their propensity to migrate and invade. Demethylation *in vitro* is also associated with changes in the expression of the metalloproteinases involved in the metabolism of the basement membrane and the epithelial-to-mesenchymal transition and tumour metastasis, notably MMP2 and TIMP2. Changes in expression were confirmed at transcriptomal and proteomic levels in response to demethylation. *In vivo*, MMP2 expression was shown to be statistically significantly associated with EMVI, DFS, and OS, and was also independently predictive of EMVI, raising the possibility that it could act as a diagnostic and predictive biomarker in rectal cancers.

These findings indicate a mechanistic association between hypermethylation and EMVI, mediated by methylation-dependent expression of metalloproteinases. Metalloproteinase expression, specifically MMP2, may act as a potential biomarker for EMVI and correlates to survival outcomes in rectal cancer.

Hypothesis Cycle & Chapters

A Mechanistic Investigation of the Relationship Between Extramural Vascular Invasion (EMVI) and CpG Island Methylator Phenotype (CIMP) in Rectal Cancer



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Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Published Papers & Presentations Associated with this Thesis

Papers

- **Kokelaar, R.F**., et al., Locally advanced rectal cancer: management challenges. Onco-Targets Therapy, 2016. 9: p. 6265-6272.
- Jones, H., **Kokelaar, R.F.,** et al., Perineal reconstruction and surgical complication delay adjuvant chemotherapy after surgery for rectal cancer but do not impact upon survival. Diseases of the Colon & Rectum, 2017. 60(6): p. e47-e593
- **Kokelaar, R.F.,** et al., DNA hypermethylation as a predictor of extramural vascular invasion (EMVI) in rectal cancer. Cancer Biology & Therapy, 2017: p. 1-8.
- **Kokelaar, R.F.,** et al., The role of hypermethylation in radiosensitivity of colorectal cancer - an in vitro model. British Journal of Surgery, 2017. 104: p. 7-57.
- **Kokelaar, R.F.,** et al., Meta-analysis of the prognostic value of CpG island methylator phenotype in rectal cancer. International Journal of Colorectal Disease, 2018. 33(8): p. 995-1000.

Presentatins (oral international / national only)

- **Kokelaar, R.F**., et al. Epigenetic and biological effects of 5-azacytidine and RRX-001 on DLD-1 colorectal cancer cell lines. **American Association for Cancer Research**; 2017. April 04-06, 2017. Washington DC, USA.
- **Kokelaar, R.F**., et al., Demethylation inhibits migration and invasion of DLD-1 colorectal cancer cells in vitro. Diseases of the Colon & Rectum, 2017. 60(6): p. e47-e593. **The American Society of Colon and Rectal Surgeons**; June 10–14, 2017 Washington State Convention Centre and Sheraton Seattle Hotel, Seattle, WA, USA.
- **Kokelaar, R.F.,** et al., Demethylation of DLD-1 colorectal cancer cell lines reduces tumour cell invasion in vitro, correlating with modulation of metalloproteinase expression. Colorectal Disease, 2018. 20(S7): p. 13-58. **ACPGBI Annual Meeting**, Birmingham, UK

Abbreviations

5-FU	5-Fluorouracil
ACPGBI	Association of Coloproctology of Great Britain & Ireland
AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli gene
AZA	5-Azacytidine
BMI	Body Mass Index (kg/m^2)
bp	Base pairs
cDNA	Complimentary DNA
CEA	Carcinoembryonic Antigen
CI	Confidence Interval (95%, unless specified otherwise)
CIN	Chromosomal Instability
CIMP	CpG Island Methylator Phenotype
cm	Centimetres
CMS	Consensus Molecular Subtype
CRC	Colorectal Cancer
CRT	Chemoradiotherapy
CT	Computed Tomography
DFS	Disease-Free Survival (months)
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Endothelial – Mesenchymal Transition
ERAS	Enhanced Recovery After Surgery
FAP	Familial Adenomatous Polyposis
Gy	Gray ($J\ Kg^{-1}$)
HR	Hazard Ratio
IBD	Inflammatory Bowel Disease (Crohn's & Ulcerative Colitis)
(K)RAS	(Kirsten) RAt Sarcoma oncogene
LARC	Locally Advanced Rectal Cancer
LOH	Loss of Heterozygosity
LRRC	Locally Recurrent Rectal Cancer

mAb	Monoclonal Antibody
MMP	Matrix Metalloproteinase
MMR	Mismatch Repair (Genes)
mr-	MRI-based assessment of tumour characteristic (e.g. mrEMVI)
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MSI	Microsatellite Instability
msPCR	Methylation-Specific PCR
nCRT	Neoadjuvant Chemo-Radiotherapy
p-	Histopathology-based assessment of tumour characteristic (e.g. pEMVI)
pCR	Pathologically Complete Response
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real-Time PCR
R0	Complete Macro- and Microscopic Tumour Excision
RFS	Relapse-Free Survival (months)
RR	Relative Risk
rtPCR	Real-Time PCR
SAM	S-Adenosyl Methionine
siRNA	Short Interfering RNA
TAMIS	Transanal Minimally Invasive Surgery
TaTME	Transanal Total Mesorectal Excision
TEMS	Transanal Endoscopic Microsurgery
TIMP	Tissue Inhibitor of Metalloproteinase
TME	Total Mesorectal Excision
TMA	Tissue Microaray
VEGF	Vascular Endothelial Growth Factor receptor
VTE	Venous Thromboembolism
WHO	World Health Organisation

Chapter 1

General Introduction

1.1 Colorectal Cancer

1.1.1 Structure & Function of the Colon & Rectum

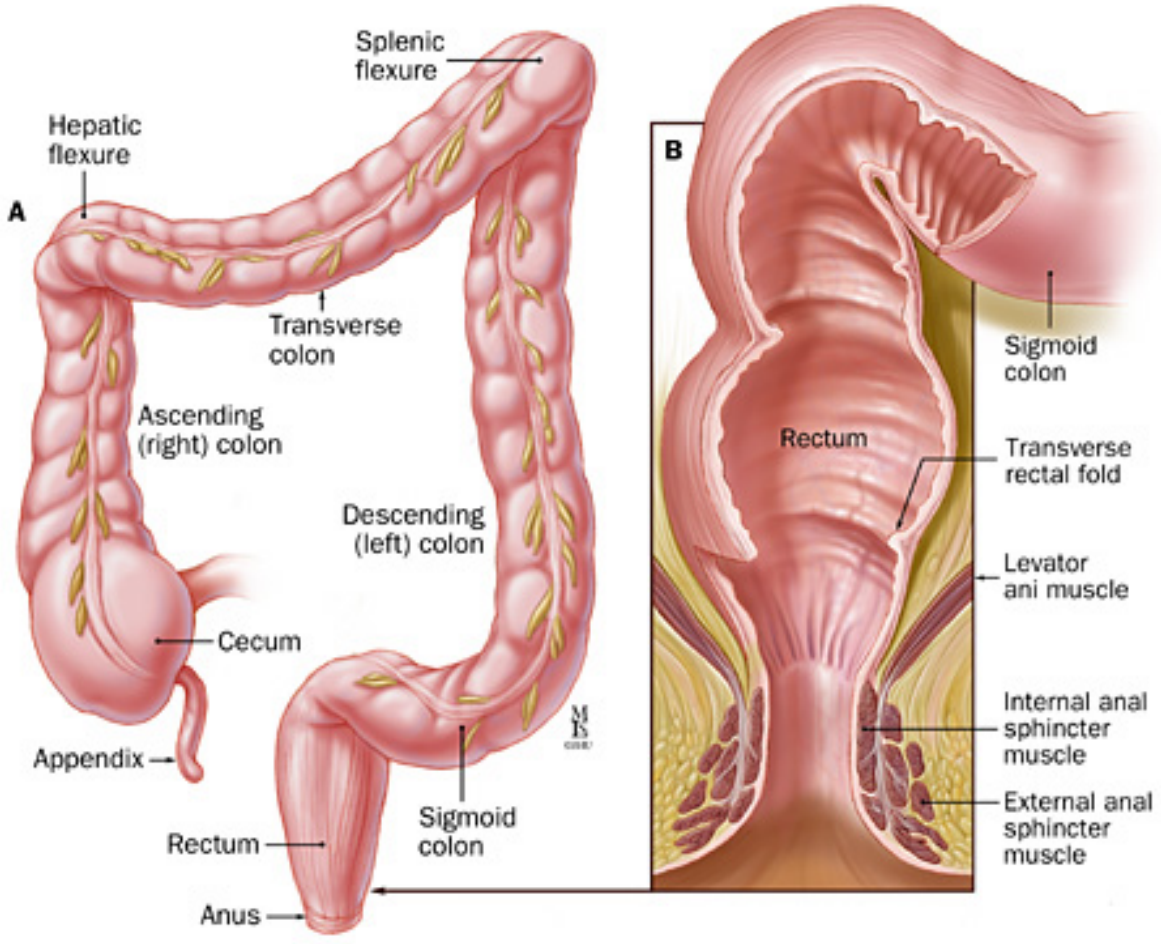
The colon and rectum constitute the large bowel/intestine, and along with the anal canal, form the final portion of the alimentary tract. Together, the colon and rectum are approximately 160cm in length, extending from the ileocaecal junction with the small bowel (marked by the ileocaecal valve) to the anal verge and skin of the perineum. The primary function of the colon and rectum is to absorb water and salts form the bowel content before it is expelled, the rectum also acting as a reservoir for stool prior to evacuation. The constituent portions of the colon are the caecum, right or ascending colon, transverse colon, left or descending colon, and sigmoid colon. The colon and rectum are comprised of concentric layers with different structure and function, the innermost of which is the mucosa (predominantly columnar epithelium with mucous producing goblet cells), followed by the neurovascular submucosa, then the muscularis propria (two layers of smooth muscle, the innermost circumferential and the outer in discontinuous longitudinal bands called taeniae coli), and finally the serosa or adventitia (depending on relationship to the peritoneum). At the distal end of the sigmoid colon the taeniae coli converge as they pass over the sacral promontory to form the rectum as a distinct anatomical portion of the large bowel.

The rectum itself is approximately 15cm in length and is divided into thirds dependent upon its peritoneal relations; the upper rectum having a peritoneal covering on three sides (anterior and bilateral), the mid rectum having only an anterior covering of peritoneum, and the low rectum being entirely below the peritoneal reflection completely enveloped in its mesentery. The classical boundary of the rectum and its supporting mesentery below the peritoneal reflections is described by the plane of complete mesorectal excision (CME), originally described in Heald's seminal work[1]. The rectum is also different from the colon in its relation to the abdominal and pelvic peritoneal cavity; being entirely located within the confines of the bony pelvis. This is of particular importance when considering both the relative limitations of

surgical access to the rectum compared to the colon, and its relationship to significant visceral, neurovascular, bony, and other structures located in the pelvis.

As the rectum reaches the pelvic floor the smooth muscular fibres constituting its outer wall condense, and with the contribution of the skeletal muscles of the adjacent pelvic floor, the anal sphincter complex is formed, investing within it the anal canal. The canal itself is 2-4cm in length, and as well as its role as a sphincter, is also marked by the change from the columnar epithelium of the colon and rectum to the squamous epithelium of the skin at the pectineal line. Due the different nature of its epithelium, the anus possesses pathology that is specific to itself and quite different to that of the colon and rectum, especially when considering carcinogenesis, and thus is outside the remit of this thesis.

Figure 1.1 The Colon (A) and Rectum (B).



The blood supply to the colon and rectum is primarily comprised of terminal branches of the superior mesenteric artery (which also supplies the majority of the small bowel) and the inferior mesenteric artery, both of which originate from the abdominal aorta. Classically, the watershed between these two arterial systems is defined as the transition from mid-gut to hind-gut, two thirds of the way across the transverse colon where the marginal artery forms an arcade connecting the two. A third arterial supply is also provided by the middle and inferior rectal arteries, which are terminal branches of intermediate arteries originating from the internal iliac artery. The venous drainage of the colon and rectum reflects the arterial in its peripheral distribution, but instead of returning blood to the systemic circulation directly, blood is diverted through the liver first via the portal venous system. The exception to this is in the low rectum where the haemorrhoidal veins return venous blood to the systemic circulation directly via the internal iliac veins. The lymphatic drainage mirrors the arterial system and comprises the superior and inferior mesenteric lymph node chains, which themselves drain to the pre-aortic nodes at levels corresponding to the origin of the arteries.

As well as absorbing water and forming and storing stool, the colon and rectum allow the bacterial fermentation of residual organic matter and act as a reservoir of bowel flora; the colorectal microbiome. Although the colon does not play an active role in absorption of nutrients, it does however absorb some of the products of bacterial fermentation, importantly fat-soluble vitamin K, a key factor in blood coagulation and calcium metabolism. Beyond fermentation, the colorectal microbiome is attracting significant interest as a potential factor in cancer and other (particularly inflammatory) processes in the large intestine, both from a pathogenic and potentially therapeutic perspective[2, 3].

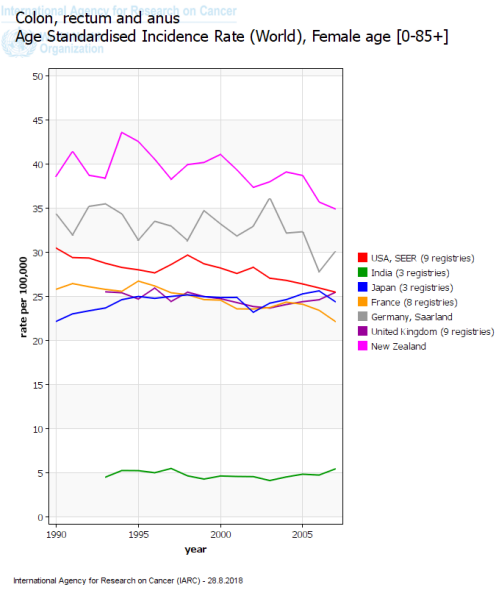
1.1.2 Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is a highly prevalent cancer in Western societies, with approximately 140,000 new cases and 52,000 deaths (estimated) in the United States in 2018[4]. In the UK the figures are 42,000 and 16,000, respectively[5]. Globally CRC is the third most common cancer in men (10.0% of total cancer) and the second most common cancer in women (9.2% of total cancer). Approximately 45% of those diagnosed die of the disease despite treatment[6]. The overall lifetime risk of developing colorectal cancer is 1 in 22 (5.5%) for men and 1 in 24 (5.15%) for women in the United States, although incidence is highly variable internationally. The incidence of CRC per 100,000 ranges from 4.1 (India) to 49.1 (Czech Republic) in men, and from 3.9 (India) to 39.5 (New Zealand) in women, with broadly higher incidence in North America, Europe (including ex-Soviet nations), and Oceania, and lower incidence in South America, Africa, and Southern Asia[7]. Although incidence had generally remained consistent in most developed nations, mortality is slowly declining, although this is primarily observed in long-industrialised nations with developed healthcare systems (**Figure 1.1 a-d**).

Although the incidence of CRC is highest in older populations (median age at diagnosis is 70 years), it may affect any age, although is uncommon under the age of 50 years. The increased risk of CRC in later life is the primary factor in the design of the national bowel screening programme in the UK, which offers screening in the form of faecal occult blood testing to both men and women over the age of 60 years, or a one-off colonoscopy to those of 55 years (regional variation dictating). The age at which screening begins is however under current review and is likely to decrease to 50 years in the near future. Screening ceases at 74 years of age. By 2012, the UK screening programme had identified almost 15,000 cancers, of which a significantly higher proportion were found at an earlier stage than in the un-screened population[8]. Uptake of screening is generally good (approximately 55%), and an up to 18% reduction in mortality has been reported[9].

Figure. 1.2 Incidence (a & b) and mortality (c & d) of CRC (male and female) – selected countries. WHO International Agency for Research on Cancer: Global Cancer Observatory (http://gco.iarc.fr) (accessed 28th August 2018).

a.



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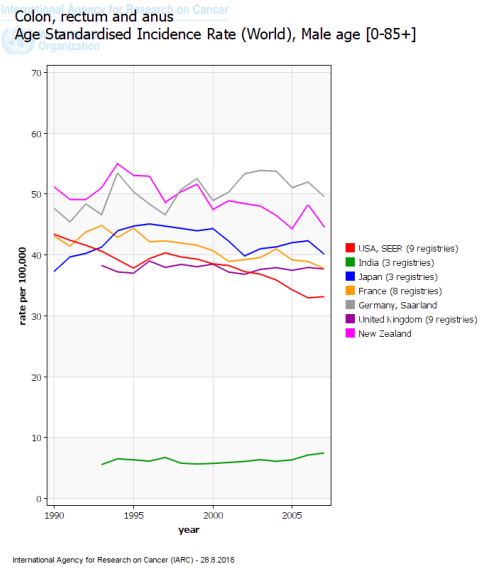

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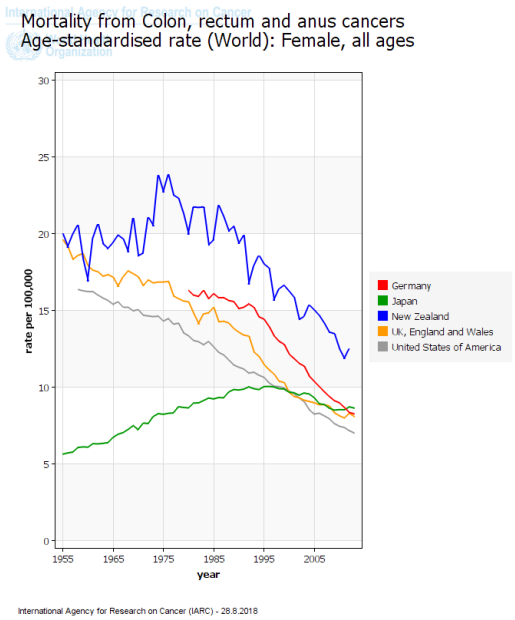

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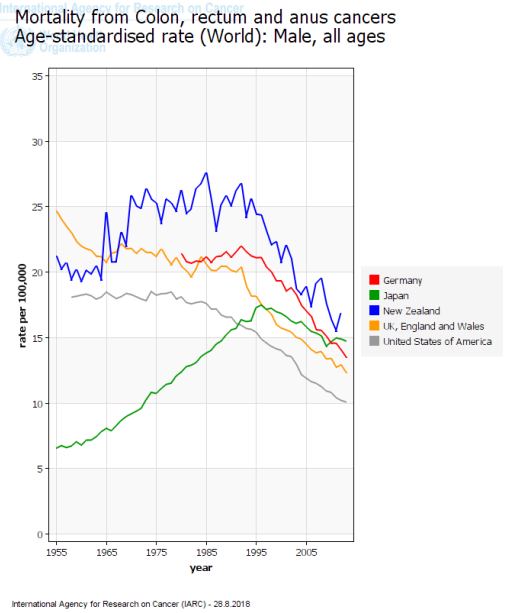


1.1.3 Aetiology of Colorectal Cancer

The primary unmodifiable risk factor in CRC is age; the median age at diagnosis being 70 years in Western societies[10]. The association with increased age is due to cumulative generalised DNA and cell damage that occurs during ageing, although specific aberrations at the genomic, epigenomic, transcriptomic, and other mechanistic levels are constantly being hypothesised, tested, and proposed. Colorectal cancers affecting younger patients are more frequently encountered in the hereditary cancer syndromes, which account for 2-5% of all CRC[11]. These syndromes are frequently characterised by the presence of polyps and are associated with highly penetrant inherited mutations and clinical syndromes, such as Lynch syndrome, familial adenomatous polyposis, MUTYH-associated polyposis, and hamartomatous polyposis. The detection of such syndromes through familial screening, given the high risk of CRC, frequently prompts prophylactic colectomy, although a significant proportion present sporadically[12]. As well as the small percentage of hereditary CRC associated with specific syndromes, it is estimated that a further 20-30% of CRC is linked to heritable mutation(s), although the loci are not characterised[13].

A further significant non-modifiable risk factor for the development of CRC is inflammatory bowel disease (IBD: Crohn's and ulcerative colitis), although the incidence has been reducing in recent years. The pathogenesis of CRC in IBD is due to chronic inflammation of the colon and rectum, and thus anti-inflammatory therapies for IBD are effective at reducing the lifetime risk of developing cancer. Correspondingly, the severity of inflammation, length of exposure, and extent of colitis are all positively correlated with risk of CRC[14]. For ulcerative colitis the incidence is approximately 3 cases of CRC per 1000 patient-years (0.3% annually), corresponding to approximate risk of 2%, 8%, and 18% at 10, 20, and 30 years disease-exposure, respectively[15]. The corresponding risk in Crohn's disease is 3%, 5.5%, and 8%, respectively[16].

Beyond non-modifiable risk factors, it is estimated that 54.3% of CRC is preventable due to population-level exposure to numerous modifiable risk-factors[17]. Foremost amongst these factors are smoking, obesity, lack of dietary fibre, and consumption of red/processed meat; contributing 6.8%, 11.5%, 28.0%, and 12.5% to the excess cancer burden, respectively, based upon a population attributable fractions analysis. Alcohol (dose dependent[18]), ionising radiation, and insufficient exercise contributed smaller but measurable effects.

The risk of developing CRC from smoking is directly proportionate to the number of cigarettes smoked per day, the number of years an individual has smoked, and the pack-year metric; and is measurable as a relative risk (RR) based on meta-analysis as 1.20 (95% confidence interval (CI): 1.10-1.30), compared to never-smokers. The effect is greater in men than women (RR 1.38 vs 1.06) and is persistent following cessation of smoking[19]. Smokers (RR 1.26) and former-smokers (RR 1.11) also suffer a significantly higher all-cause mortality following a diagnosis of CRC compared to never-smokers, based on a random effects hazard ratio model[20].

Obesity (BMI ≥30), and to a lesser extend overweight (BMI ≥25.0-29.9), are independent risk factors for CRC, especially in men. Of particular concern is visceral/abdominal obesity, where each increase in BMI of 1 kg/m^2 infers an increased risk (RR 1.03)[21]. Various mechanisms are suggested, including metabolic syndrome and the effects of insulin resistance, and dysregulating of adipocytokines. Hand-in-hand with obesity, type-2 diabetes has also been demonstrated to show an increased risk of CRC (Hazard Ratio (HR) 1.26, CI 1.18-1.33), with risk increasing with duration of risk-exposure[22].

The role of dietary fibre in reducing the risk of colorectal cancer has long been the subject of speculation, and a number of mechanisms have been postulated, including bulking and dilution of luminal toxins, decreased transit time, and short-chain fatty acid metabolism of fibre by the microbiome, amongst others[23]. A meta-analysis of dietary fibre intake and risk of developing CRC demonstrated that there was an inverse correlation between increased fibre intake and risk, and that this correlation was dose dependent (10% RR reduction per 10g/day total dietary fibre)[24]. The effects were observable for total dietary fibre, wholegrain fibre, and cereal fibre, but no association was discernible with fibre sourced from fruit, vegetables, or legumes. The authors of this interesting analysis suggest that the bulking and decreased transit time associated with cereal fibre may be causal in this relationship, although they do admit that there is likely to be an element of confounding due to the insulin-sensitising and excess-weight preventing effects of a high-fibre diet.

Consumption of red (fresh) and processed meat is associated with an increased risk of developing CRC, although the data is somewhat heterogeneous[25]. In a recent meta-analysis, consumption of one portion of processed red meat per day was associated with a pooled increase in CRC risk (HR 1.15, CI 1.01-1.32), but this increased for tumours located in the distal colon and rectum (HR 1.36, CI 1.09-1.69)[26]. Interestingly, the data for fresh red meat

suggested a reduced risk of distal cancers (HR 0.75, CI 0.69-0.82), but a possible increased risk for proximal cancers (statistically non-significant). Although the mechanism for these effects remains contentious, the addition of nitrates and nitrites in processed meat, and the increase in mutagenic heterocyclic amines caused by cooking (and especially burning) meat are proposed as the most likely mechanisms[27, 28].

1.1.4 Staging and Histopathological Standards in Colorectal Cancer

Current standards for the staging of colorectal cancer are set by the American Joint Committee on Cancer (AJCC), 8th Edition[29, 30], and were formally adopted for practice in the UK from January 2018 (the previous 2 editions having been bypassed due to lack of supporting evidence)[31]. Broadly, tumours are staged dependent on their depth of invasion through the bowel wall (T stage), by lymph node spread (N stage), and metastatic spread (M stage). A composite of these three scores (TNM) is then used to provide an overall Stage (I-IV), which is intended to stratify survival related to the disease. The gold-standard for staging is histopathological examination of the resected tumour, although at different stages of the patient's treatment staging may be performed using other modalities (such as MRI), or following therapies (such as neoadjuvant chemo-radiotherapy). To aid in differentiating how staging has been performed a prefix may be added to the TNM system to indicate modality (e.g. *p*: pathological staging; *c*: clinical staging), or to indicate whether therapies have been applied (e.g. *yp*: pathological staging post chemo-/radiotherapy).

Historically, the Dukes' and Bussey Classification (Stages A-C) has been used to characterise the local and nodal extent of colorectal cancer, with the later addition of Stage D by Turnbull to denote metastatic spread[32, 33]. This system is still advocated for use in parallel with the AJCC classification by the Association of Coloproctology of Great Britain and Ireland (ACPGBI) in their current (2017) guidelines for the management of cancer of the colon, rectum, and anus[34]. To aid in the classification of colorectal cancers it is important that strict standards are set in the reporting of histopathological specimens. This is crucial not just in determining the extent of an individual's disease, and therefore their management, but also for audit, research, and strategy for the local, national, and international management of colorectal cancer. To this end a minimum-reported dataset is published by the Royal College of Pathologists (latest edition 2017), setting standards for histopathological examination of resected specimens[35]. As well as a macroscopic description of the tumour and the basic tumour characteristics that comprise the TNM classification system, the core dataset also includes information that may aid in

prognostic stratification, such as the degree of tumour differentiation, the presence of mucin, and extra-mural and peri-neural invasion. Estimation of tumour regression following neoadjuvant therapy is also included in the minimum dataset (where appropriate) and is based upon the four-tier system (0-3) proposed by Ryan[36]. Increasingly, genetic and molecular biomarkers are also employed in the characterisation of colorectal cancers, and routine testing for mismatch repair (MMR) status (see 1.3.4) (and subsequently BRAF V600 E and/or MLH1 analysis) to distinguish sporadic cancers from Lynch Syndrome is now considered part of the core dataset for reporting colorectal cancers[37]. Additionally, testing targeted at genes associated with response to EGFR therapies (KRAS and NRAS codons) may be performed in patients with metastatic colorectal cancers to identify those who may benefit from treatment[38].

1.1.5 Survivorship and Outcomes in Colorectal Cancer

Survival following diagnosis and treatment for colorectal cancer is primarily dependent on the stage at which the cancer was diagnosed, with earlier stage cancers translating into improved survivorship; and is the premise of colorectal cancer screening programmes. Approximately 80% of individuals diagnosed with colorectal cancer undergo surgery[39]. Data form the American Cancer Society suggest that the 5-year survival from Stage I colon and rectal cancer is approximately 92% and 88%, respectively, falling to 12% and 13% respectively for Stage IV cancer[40]. This data is summarised in **Table 1.1**. Patients who are elderly, present as an emergency, or have a high number of comorbidities are at an increased risk of dying compared to the background risk[10, 41]. For patients with Stage III or high-risk Stage II disease, the addition of adjuvant chemotherapy to surgery increases 5-year survival across the population by 5%, although we currently lack the ability to predict which individuals will benefit. Unfortunately, recurrence following treatment with curative intent in colorectal cancer is approximately 45%, of which only 5-10% of patients may be eligible for further "curative" surgery[42].

Beyond staging, the factors affecting the risk of recurrence are not well understood. Follow-up after treatment with curative intent is aimed at identifying recurrence early (especially lung and liver metastasis), and thus increasing the chances of surgical management or eliciting a better response to chemo- or immunotherapy. The detection of metachronous tumours and polyps is also facilitated by follow-up, as well as providing psychological and social support to potentially vulnerable patients[43]. There is some variation in the protocols for following-up patients, but a combination of CEA and CT-scanning at 6-monthly intervals initially, and then declining over time, is the mainstay of assessment, augmented with colonoscopy or other

imaging modalities where necessary[44]. Recent meta-analysis has demonstrated that more intensive follow-up regimes have shortened the time to detection of recurrence by up to 10 months, although this has so far failed to translate into statistically significant population survival benefit[45]. Effective follow-up is also important for MDT and audit purposes, is key to delivery of many of these services in the Colorectal Clinical Nurse Specialist, and is reassuring for patients[46].

Table 1.1. 5-year survival following diagnosis of colon or rectal cancer, by AJCC Stage[40].

AJCC Stage	**I**	**IIA**	**IIB**	**IIIA**	**IIIB**	**IIIC**	**IV**
Colon	92%	87%	65%	90%	72%	53%	12%
Rectum	88%	81%	50%	83%	72%	58%	13%

1.2 Treatment for Colorectal Cancer

1.2.1 Surgery for Colorectal Cancer

The primary therapy for colorectal cancer is surgery, with approximately 80% of patients diagnosed with CRC undergoing surgery with curative intent. The surgical strategy employed is based upon the anatomical location of the tumour(s) and the associated lymphovascular package. Segmental resection of the affected colon or rectum in association with its draining lymph nodes and vasculature reduces the local recurrence by excising the primary tumour plus any local macro- or micro-metastasis. Broadly, tumours of the right colon require right hemicolectomy, tumours of the left colon a left hemicolectomy or sigmoid colectomy, and tumours of the rectum an anterior or abdominoperineal resection. For metachronous tumours affecting the left and right hemicolons, or where a heritable or sporadic pro-carcinogenic field-change in the colonic mucosa is suspected or proved, excision of larger portions of or the whole colon (with or without the rectum) may be performed as a subtotal or total colectomy (or panproctocolectomy). If a tumour has invaded structures/organs beyond the normal plane of excision (T4), these may be amenable to resection *en bloc*. In the majority of cases, following resection of the affected colon or rectum, an anastomosis may be formed between the remaining proximal and distal bowel in order to restore gastrointestinal continuity, although a temporary or permanent ostomy may be constructed depending on a number of technical or patient factors.

Although the enduring principles of surgery for colonic cancer were laid down by Cuthbert Duke and Lockhart-Mummery at St Marks Hospital in the 1950's, and standards for rectal cancer surgery by Heald in 1982 in Basingstoke, recent advances in technique and perioperative management have improved outcomes for patients. Enhanced recovery after surgery (ERAS) programmes have been demonstrated to be safe and effective in reducing perioperative morbidity and reducing the length of hospital stay following surgery[47]. The administration of carbohydrate-rich drinks immediately prior to surgery has also been shown to reduce insulin resistance and facilitates gastrointestinal recovery[48]. The role of mechanical bowel preparation is still controversial, but a growing body of evidence suggests that it is unnecessary in the majority of situations, with rectal resection and the formation of a (low) colo-rectal anastomosis being the possible exception[49, 50]. However, as the role of the colorectal microbiome becomes better understood, and intraoperative fluorescence technologies become more widely adopted, the role and nature of mechanical (or microbiomal) bowel preparation will likely change[51, 52].

Laparoscopic techniques now have an accepted role in surgery for CRC. Laparoscopic resection of colonic cancers has been demonstrated to be equivalent to open resection in terms of R0 resection rate and nymph node harvest, but superior in terms of length of hospital stay, post-operative pain, wound healing, wound infection, blood loss, and risk of incisional hernia. The Association of Coloproctology of Great Britain and Ireland (ACPGBI) guidelines for the surgical management of colorectal cancer support the implementation of laparoscopic techniques in all suitable cases (recommendation grade A)[53]. The case for laparoscopic surgery in rectal resection is less well evidenced. Although many of the same short-term advantages of laparoscopy may be apparent as for colonic resection, the ALaCaRT and ACOSOG Z5061 trials failed to demonstrate non-inferiority of laparoscopic surgery compared to open for core pathological criteria[54, 55]. Despite these rather disappointing findings, the data is deemed insufficiently significant to prevent the application of laparoscopic techniques to rectal resection, and the ACPGBI support its implementation (recommendation grade B)[53]. As yet, there is no substantial evidence to support the use of robotic surgery in either routine colorectal cancer surgery or selected challenging rectal cancer cases, although long-term outcome data still awaited[56].

1.2.2 Emergent Surgical Strategies for Rectal Cancer

Surgery for rectal cancer is increasingly being considered as a highly sub-specialised practice within general colorectal surgical practice. The challenges of operating on the rectum due to the confines of the bony pelvis and close proximity of significant neurovascular structures (e.g. iliac vessels and major branches, sciatic and obturator nerves) and other organ systems (e.g. male or female urogenital systems) requires specialist expertise and increasingly specialised techniques.

For large polyps and early cancers (T1 and early T2) transanal techniques such as transanal endoscopic microscopic surgery (TEMS) and transanal minimally invasive surgery (TAMIS) have increased the options available for surgical excision and organ preservation[57, 58]. Currently there is no consensus regarding whether one technique is superior to the other due to a lack of controlled trials and limited adoption, but early outcomes suggest that either technique is oncologically acceptable compared to traditional rectal excision[59, 60]. For patients with more advanced disease that require formal rectal resection, but who may be predicted to be operatively challenging due to a low tumour position, narrow (usually male) pelvis, or obesity, a transanal total mesorectal excision (TaTME) may be performed with laparoscopic abdominal

assistance. The aim of this technique is to perform the most challenging component of the procedure transanally, thus improving oncological resection and facilitating laparoscopic resection with the benefits of a faster return to GI function, shortening length of hospital stay, and lower postoperative morbidity[61, 62]. Currently the evidence base for an advantage in performing TaTME is poor, although data submission to the centralised international registry is designed to alleviate this issue[63, 64].

Locally advanced rectal cancers (LARC) and locally recurrent rectal cancers (LRRC) pose additional challenges to surgeons as they extend beyond the planes of classical TME. As successful surgery is dependent upon an R0 resection it is frequently necessary to resect adjacent structures that are not typically included in a standard rectal resection, either as partial composite resections (e.g. portions of the posterior wall of the vagina, sections of sacral nerve, or anterior table of sacral vertebrae), or as whole organs (e.g. total pelvic exenteration)[65, 66]. Evolving techniques in surgery (resectional and reconstructive), as well as improved perioperative care (physiotherapy, clinical psychology, etc) are driving advances to increasingly extensive resections[67, 68]. Again, due to limited numbers of patients undergoing beyond-TME excisions for LARC and LRRC the evidence base is not strong, and thus international collaborative work is aimed resolving this deficiency[69].

The converse side of the management paradigm from exenteration is the possibility for organ preservation and watch-and-wait strategies, largely brought about due to advances in neoadjuvant therapies[70, 71]. Up to 30% of patients treated with nCRT may experience a complete pathological response (pCR) and benefit from a watch-and-wait strategy of no-surgery and close observation, and for those who do not undergo pCR but do undergo downsizing/down-staging an organ preserving surgery such as TEMS or TAMIS may be appropriate[72-74]. Currently, however, there is no reliable method of predicting which patients will respond to nCRT before it is administered and there is no level I evidence supporting a watch-and-wait strategy. Many patients will not respond or progress during nCRT treatment, some becoming technically inoperable or medically unfit for surgery, and for those who do have a good response there is much debate as to the best interval between therapy and surgery[75]. The current basis for predication and assessment of response is MRI, although PET-CT and endorectal ultrasound (ERUS) also have a role[36]. Additional tumour and patient factors including new molecular means of stratification of risk and response are being sought[76, 77].

1.2.3 Chemotherapy for Colorectal Cancer

Chemotherapy has three roles in the management of colorectal cancer; prior to surgery to increase the operability of tumours and outcomes following surgery (neoadjuvant chemotherapy), following surgery with curative intent to reduce the chance of recurrence (adjuvant chemotherapy), and in the metastatic or palliative setting.

The role of neoadjuvant chemo-radio therapy (nCRT) in rectal tumours where the resection margin is threatened or breached is established[78-80]. Data from the FFCD 9203 trial demonstrated that local recurrence rates were halved from 16.5% to 8.1% ($p<0.05$) and pCR improved from 3.4% to 11.6% ($p<0.05$) with the addition of neoadjuvant chemotherapy to radiotherapy, although overall 5-year survival was not improved[79]. However, the NSABP R-03 trial demonstrated that an improvement in DFS was achievable, with trend towards increased OS[81]. There is currently a degree of variation between guidelines for administration of nCRT, although long-course 5-fluorouracil (5-FU) or capecitabine neoadjuvant chemotherapy combined with radiotherapy (45-50.4 Gy) is the basis of most protocols. Patients deemed at lower risk or unsuitable for long-course therapy may be offered short-course protocols based on local guidance, although PCR rates are lower in this group[82]. The role of adjuvant CRT following nCRT is contentious, especially given the EORTC 22921 trial of over one thousand patients, where no DFS or OS advantage was observed with the addition of CRT following neoadjuvant therapy[83]. Currently there is no role for neoadjuvant chemotherapy for the management of colon cancer, although this is the subject of ongoing clinical trials led by the FOxTROT Collaborative[84]. Early outcomes from this study suggest that for locally advanced but operable colon cancers (T3/4), neoadjuvant chemotherapy and sensitivity-specific immunotherapy improves 2-year recurrence rates (personal communication – unpublished).

Adjuvant chemotherapy for colorectal cancer should be offered to all patients with high-risk Stage II and all Stage III disease who have undergone surgery with curative intent to reduce the chance of local and systemic recurrence[85]. The current guidance offers a choice of a six-month course of capecitabine as monotherapy, or folinic acid with 5-FU and oxaliplatin (FOLFOX), to begin within six weeks of the date of surgery[86]. A pooled analysis of randomised trial data for both regimes (including DFS as well as relapse-free survival (RFS)) indicates an increase in DFS at 5-years from 42% to 58%, and an increase in OS from 51% to 64%, compared for surgery alone for stage III colon cancer. The same regimes are employed

for high-risk stage II and stage III cancers of both the colon and rectum, despite the majority of evidence pertaining to stage III colon cancers. The choice between regimes is determined between the patient, clinician, and local protocols, as there is not significant evidence for superiority between regimes on either a clinical effectiveness or cost effectiveness analysis, although the primary cited evidence is limited to only three trials (X-ACT, MOSAIC, and NSABP C-07)[87-89]. Capecitabine is a precursor of 5-FU and can be administered orally in tablet form or by intravenous infusion. Its primary mechanism of action is as an inhibitor of thymidylate synthase and thus preventing the synthesis of thymidine; a nucleotide required for DNA replication. As this action is not cancer-specific other rapidly regenerating tissues such as the normal gastrointestinal mucosa may be affected, resulting in common side effects such as diarrhoea, abdominal pain, nausea and vomiting, and mucositis. More rarely hand-foot syndrome and myelosuppression may occur. Each of these side-effects may be dose-limiting, and administration is contraindicated in myelosuppression and severe hepatic or renal impairment. Oxaliplatin is a platinum-based agent administered by intravenous infusion that prevents DNA replication by causing DNA cross-linking. As well as myelosuppression and gastrointestinal side-effects, the most common side-effect of oxaliplatin is peripheral neuropathy, which may have a cumulative effect over the course of multiple administrations.

The chemotherapy treatment options for patients with locally advanced and/or metastatic colorectal cancer, whether potentially operable or not, are multiple and therefore local protocols and patient preference determine practice[85]. First- and second-line regimes combine FOLFOX protocols with irinotecan as a single agent or in combination with 5-FU (FOLFIRI). Capecitabine and oxaliplatin in combination may also be used as a first-line therapy, followed by FOLFIRI. Irinotecan is a plant-derived cytotoxic alkaloid that inhibits both DNA replication and transcription by interference with the topoisomerase-1 enzyme, which is responsible for regulating DNA double-helix winding during these processes. For patients intolerant of 5-FU based regimes raltitrexed may also be considered, its action similarly focused on thymidylate synthase although by a different action and having a different side effect profile[90]. Tegafur (a 5-FU pro-drug) is also licensed for use as a first-line therapy in advanced colorectal cancer but is not normally preferred over FOLFOX or capecitabine regimes.

1.2.4 Biological Therapies for Colorectal Cancer

Patients with metastatic colorectal cancer may benefit from monoclonal antibody (mAb) therapy if they over-express epidermal growth factor receptor (EGFR) and are (K)RAS (Kirsten RAt Sarcoma oncogene) wild-type[91]. Both cetuximab (chimeric m-IgG1) and panitumumab (human recombinant m-IgG2) act by specifically binding EGFRs and blocking the activating ligand, thus inhibiting activation of tyrosine kinase and a cascade of intracellular signalling pathways that promote DNA synthesis and cell proliferation. Both agents in combination therapy with FOLFOX have been shown in clinical trials (OPUS and PRIME) to be superior to FOLFOX alone in terms in improved PFS and OS, although the numbers of patients benefiting from therapy were more modest than anticipated based on RAS status[92, 93]. One possible reason for mAb therapy response to correspond poorly to RAS status is the discovery of an increasing number of RAS mutations that are outside the standard analysis (RAS G13D and NRAS, for example), thus leading to unexpected EGFR therapy resistance. These findings have affected several recent trials and may steer management towards a smaller proportion of patients being eligible for mAb therapy, but with a higher response rate[94]. Two further mAb, bevacizumab and aflibercept, which are targeted towards the vascular endothelial growth factor receptor (VEGF), have also reported outcomes that do not indicate superiority to more conventional regimes and are thus not recommended by NICE for patients with metastatic CRC, although they remain licensed[95-98].

1.2.5 Radiotherapy for Colorectal Cancer

Radiotherapy in the context of colorectal cancer is predominantly focused on the neoadjuvant treatment of locally advanced or locally recurrent rectal cancer[65]. Long-course neoadjuvant chemoradiotherapy (CRT) has been the established preference for T3/4 N1/2 rectal cancers for over 20 years, and in association with TME offers the best chance of complete excision and increases OS & DFS[81, 99]. Strategies aimed at increasing the dose and accuracy of pre-/intra-operative radiotherapy have been trialled, notably in the form of rectal brachytherapy and intra-operative radiotherapy, although results have not been conclusive and currently these practices are largely confined to trials[100-102]. There is however a shifting attitude towards radiotherapy for LARC with the advent of increasingly effective chemotherapy and immunotherapy regimes[103]. One possibility is that chemotherapy alone may be as effective as CRT, and thus negate the requirement for radiotherapy altogether, theoretically reducing the risk of poor wound healing, anastomotic breakdown, and more challenging operating planes, and is the subject of the ongoing PROSPECT trial[104]. Another perspective is that radiotherapy should be

applied in the neoadjuvant setting only after neoadjuvant chemotherapy has been administered and had an opportunity to cause tumour involution; a methodology referred to as induction chemotherapy. This approach is currently undergoing investigation in a number of different trials, including the E-LARC Study and UK BACCHUS trial[105, 106]. Radiotherapy may also be employed in the palliative setting, especially where symptoms such as per-rectum bleeding are distressing to patients, or for LRRC where the pelvis has not previously been irradiated.

1.2.6 Associated Therapies in Colorectal Cancer

In addition to surgery and clinical- and medical-oncological therapies, a range of other strategies may be employed to treat CRC. Colonic stents are becoming an increasingly versatile tool in combating colorectal cancer, especially in the frail and elderly, palliative, or emergency setting (up to 20% of presentations) as a bridge to definitive therapy. Early outcomes form the CReST trial found that mortality and length of hospital stay were no different between emergency surgery and stent groups, but that the rate of permanent stoma formation was significantly lower in the stent group (45% vs 69%, $p<0.001$)[107]. Oncological outcomes are awaited. The Enhanced Recovery After Surgery (ERAS) programme has also helped deliver improvement in perioperative care, especially with respect to facilitating a reduced time to normal gastrointestinal function, early return to mobility, and reduced length of inpatient admission. These improvements have been won by standardising care as much as possible, improving patient education, early enteric feeding, reducing unnecessary drain and nasogastric tube use, early mobilisation, optimising perioperative pain management, and auditing of practices[47]. Carbohydrate loading, optimisation of the use of blood products, advanced stoma care, and venous thromboembolism prophylaxis (VTE) are also important considerations in delivering high quality perioperative care and all are included in current management guidelines[53].

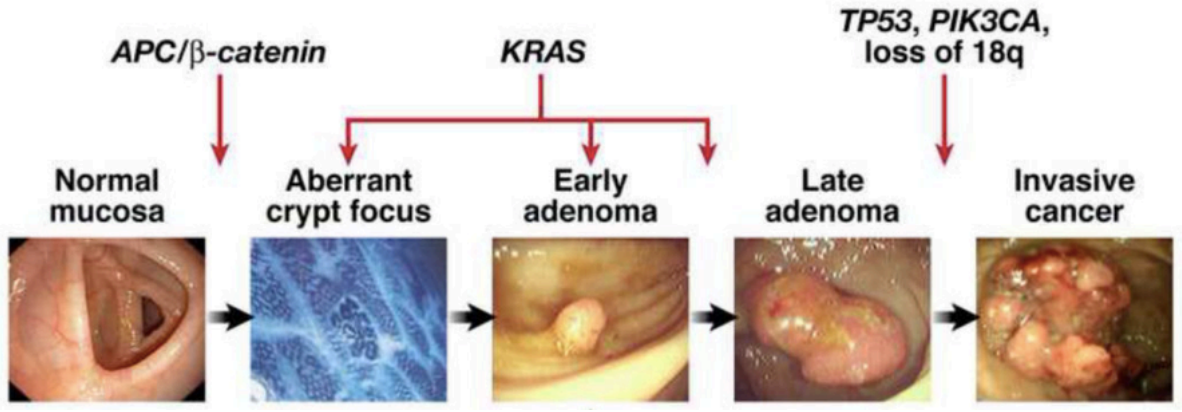
1.3 The Genetic & Genomic Basis of Colorectal Cancer

1.3.1 The Vogelstein Model

The development of sporadic colorectal cancer has long been considered as a step-wise progression along the adenoma-to-carcinoma pathway, and the genetic processes underlying this sequence were formulated by Vogelstein[108, 109]. The basis of this model is that sequential mutation in the adenomatous polyposis coli (APC) gene, and subsequent KRAS and TP53 mutations leads to increasing adenomatous changes in the colonic mucosa, resulting in polyps and eventually to dysplasia and invasion.

The first sequence in this pathway is brought about by APC mutation and subsequent dysregulation of the Wnt/β-catenin canonical signalling pathway. APC mutation leads to inappropriate intracellular accumulation of β-catenin due to inactivation of the destruction complex, resulting in activation of transcription factors governing the cell cycle. Mutations in this pathway are detected in up to 90% of sporadic colorectal cancers, and specific autosomal-dominant inherited mutations in APC are responsible for familial adenomatous polyposis (FAP)[110]. Secondly, KRAS is a proto-oncogene that regulates the cell cycle by activating growth factors, cell signalling, and glucose transportation via the MAPK-pathway. When mutated, deactivation and subsequent negative-signalling is disrupted leading to increased signalling and uncontrolled cellular proliferation[111]. The important role of KRAS in CRC therapy is underscored by its impact on the efficacy of EGFR immunotherapies as discussed in **Section 1.2.4**, although the relatively high proportion of patients with advanced cancers who are KRAS wild-type demonstrates that it is not a necessary mutation in the classical pathway. Thirdly, the role of the heterozygosity in TP53 gene (and its related protein p53) is established in tumour suppression, where its acts to stimulate DNA repair, arrest the cell cycle, initiate apoptosis, and moderate telomere response. Loss heterozygosity is associated with a number of different cancers, including CRC (also breast, endometrial, and renal tumours), although the association with mutation and risk of cancer is not linear[112, 113]. However, in the colorectal adenoma to carcinoma pathway, loss of TP53 function is detected in approximately 25% of adenomas with no adverse features and 50% of adenomas with foci of invasion, underlining its likely causative contribution to carcinogenesis[114]. Additional mutations in PIK3CA (a phosphorylation-related kinase oncogene) and allelic deletion of segments of chromosome 18q encoding the DCC (deleted in colorectal carcinoma) gene (tumour suppressor) cumulatively drive carcinogenesis as a core set of oncogenic mutations[115].

Figure 1.3 The multi-step genetic model of colorectal carcinogenesis (adapted from Pino, et. al.)[115].



These cumulative genetic insults are thought to underlie many colorectal cancers, although due to the relatively short lifespan of individual colorectal mucosal cells and the low background rate of mutation, the likelihood of any single cell developing the multiple mutations required to undergo carcinogenic change is limited. What underlies the accelerated process of mutation, therefore opening the opportunity for multiple tumourigenic transformations, is loss of genomic stability[116, 117]. There are several processes thought to underlie the development of genomic instability, each of which may contribute to malignant transformation, but in the context of CRC the primary factors are microsatellite instability (MSI) and chromosomal instability (CIN)[118].

1.3.2 Key Signal Transduction Pathways in CRC

Several key signal transduction pathways play an important role in CRC carcinogenesis, and are implicated in the Vogelstein and other models of cellular dysregulation. Although frequently presented as isolated pathways, these signal transduction pathways are in fact complex webs that govern cellular homeostasis; controlling vital processes such as cellular proliferation, apoptosis, cell-cell adhesion, and metabolism. The primary genes/proteins governing signal transduction pathways in CRC are APC/β-catenin, EGFR, KRAS, and BRAF. A schematic representation of these pathways and their interdependent relationships is demonstrated in **Figure 1.3**.

Activation of Wnt signalling through APC mutation, typified by the germline mutations that define FAP, is an important initiator of CRC carcinogenesis[108]. APC dysfunction is identified in 70% of CRC, and has widespread intracellular effects, including regulation of cellular proliferation, differentiation, apoptosis, and chromosomal segregation, although its primary

pathological effect when dysfunctional is to increase cytoplasmic and nuclear β-catenin and thus drive cell proliferation and invasion through activation of T-cell factor/lymphoid enhancer factor (LEF) transcription factors[119, 120]. Downstream tumourigenic effects of increased LEF activity are modulation of tissue remodelling via the urokinase receptor, plasminogen activation system, and tight-junction proteins[120].

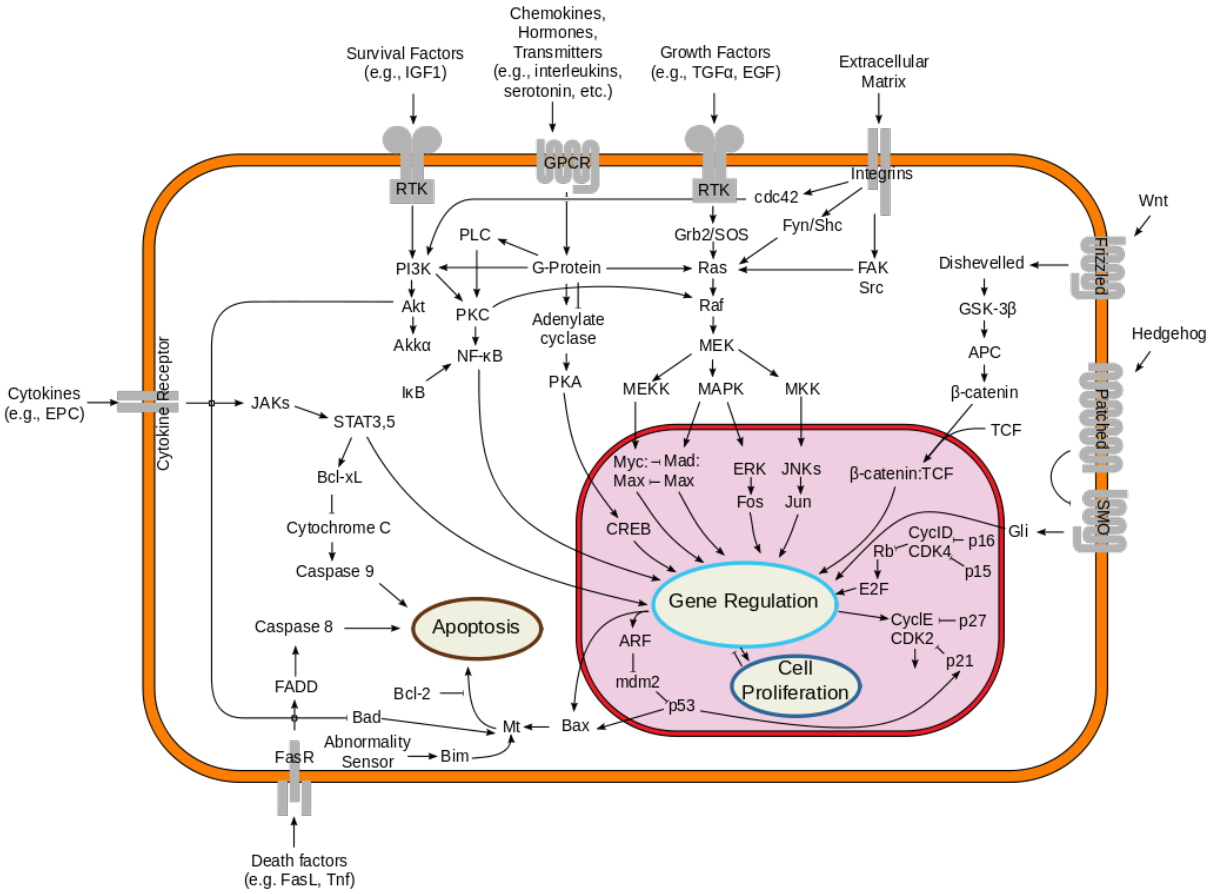
The epidermal growth factor receptor (EGFR) is a transmembrane protein which acts as a receptor for extracellular ligands, binding of which modulates intracellular signalling cascades that are intimately associated with cell proliferation, adhesion, and migration[121]. As discussed previously, EGFR has a role in the biologic therapy of colorectal cancers, the efficacy of which may be determined by KRAS and BRAF mutation. Modulation of EGFR activity, its downstream signalling pathway, and other associate genes is an active research area for novel chemotherapeutics[122-124].

The *KRAS* (Kirsten Rat Sarcoma Viral Oncogene Homolog) gene encodes a small GTPase that is primarily involved in regulating cell division, acting as an on-off switch for intracellular signal transduction via both vertical (nuclear-directed) and horizontal (cytoplasmic) pathways. The role of KRAS in CRC has long been established and forms a central tenet of the Vogelstein model of carcinogenesis[109]. When mutated, the negative (inhibitory) effects of the RAS are lost, allowing cell proliferation and inhibition of apoptosis. KRAS mutations are very common in colorectal cancers (35-40%), especially those located in the proximal colon, and form a distinct morphological subset associated with polyps, mucin formation, and MSI[125, 126]. As discussed in **Section 1.2.4**, KRAS mutations are important in determining the sensitivity of CRC to biologic therapies

BRAF is a gene located on chromosome 7q34 that encodes a 94 kDa protein called serine/threonine-protein kinase B-Raf, which acts as the common lead point for the RAS-RAF-MEK-ERK-MAP kinase signalling pathway [modulated by PKC as well as RAS][127]. As well as being associated with a poor prognosis in CRC, BRAF mutations are also associated with 15% of all human cancers, as well as some birth defects[128]. Activation of proto-oncogenes, particularly those associated with apoptosis and programmed cell death, underlies BRAF's role in carcinogenesis, and may be associated with CIMP, particularly in the frequently encountered BRAFV600E mutant[129, 130]. BRAF-dependent signalling is also a potential therapeutic target in cancers, with successful biologic therapies having been developed for treatment of melanomas[131]. Currently, there are no specific therapies directed against this pathway for

BRAF-mutant CRC, although the role of biologic agents for metastatic CRC is being explored[132, 133].

Figure 1.4 Schematic representation of APC, EGRF, KRAS, and BRAF signal transduction pathways in relation to CRC gene regulation and apoptosis. (adapted from Lodish, et. al.)[134]



1.3.3 Tumour Suppressors - TP53 & 18q

TP53 is a gene located on the short arm of chromosome 17 that codes for a corresponding protein (p53) which has a critical role in tumour suppression. The majority of mutations are missense mutations that occur in a number of common loci; leading to inactive and long-lived proteins. In response to a wide range of signals, including oxidative stress, DNA damage, and abnormal intracellular signalling (including RAS), p53 coordinates a multitude of cellular actions by initiating the transcription of genes associated with cellular metabolism, apoptosis (especially caspase regulation), cell cycle regulation, immunological signalling and response, and cell differentiation[135]. p53 mutations are widely reported in human cancers, and are present in at least 70% of invasive CRC, as well as 25% of adenomas and 50% of polyp cancers, indicating the early and likely necessary contribution to CRC formation[114].

Approximately 70% of CRC demonstrate allelic loss at chromosome 18q, although the suspected tumour suppressor genes located here have not been demonstrated to be common in CRC[136]. Examples of such genes are the unfortunately named Deleted in Colorectal Cancer gene (DCC), which is only found in 6% or tumours, and the SMAD2 (20% of tumours) and SMAD4 (10% of tumours) genes involved in TGF-β signalling[137, 138]. Loss of heterozygosity of the Cables gene, which is located on chromosome 18q, has however been associated with colorectal cancer (70% LOH), although its role in carcinogenesis is not well defined[139].

1.3.4 Microsatellite Instability & Mismatch Repair

Microsatellite Instability (MSI) describes a situation linked to defective mismatch repair (MMR) where repeating motifs of non-coding DNA are introduced during the S-phase of DNA replication, leading to cells demonstrating a high degree of mutability[140]. Mutations may take the form of single base substitutions or short insertions or deletions, which are accumulated over time to form multiple-repeated motifs known as microsatellites (typically 1-6bp, repeated up to 50 times). The most common microsatellite in human DNA is the dinucleotide repeat of cytosine and adenine (i.e. CACACACACACACA…), leading to a frame-shift mutation in the daughter strand of DNA and subsequent protein inactivity[141]. International standards for the diagnosis and classification of MSI were developed by international consensus and depend on a panel of five validated microsatellites for clinical and research use[142]. Tumours may be classified as MSI-high (MSI-H), ≥ 2/5 microsatellites; MSI-low (MSI-L), 1/5 microsatellites; or MSI-stable (MSI-S), ≤ 1/5 microsatellites but dependent of further characterisation.

Approximately 15% of CRC express MSI (12% sporadic, 3% Lynch syndrome); predominantly due to defects in the mutL homologues (MLH1 & MLH3) and mutS homologues (MSH2, MSH3, MSH6) involved in MMR[143]. CRCs expressing a high degree of MSI (MSI-H) have a slightly better prognosis compared to those with an MSI stable or low (MSI-S / MSI-L) phenotype[144]. Autosomal-dominant inherence of mutations in MMR genes (primarily MSH2, MLH1, and MSH6) characterises Lynch syndrome (Hereditary Non-Polyposis Colorectal Cancer – HNPCC), a heritable CRC syndrome which carries an 80% lifetime risk of CRC[145]. MSI is also associated with hypermethylation, particularly CpG island methylations phenotype high (CIMP), often affecting MMR genes such as MLH1, leading to silencing[146]. This form of genomic instability is most commonly associated with the serrated pathway and BRAF mutations that are more commonly observed in the right colon, although many of the gene mutations are shared with the classical adenomatous pathway found throughout the colon and rectum. Where this sub-type of tumour is found within the rectum it may represent a less favourable molecular subtype[125, 147-150].

1.3.5 Chromosomal Instability (CIN)

CIN is a process where portions of chromosomes are either deleted or amplified asymmetrically, leading to an asymmetric distribution of DNA on mitosis and subsequent aneuploidy of daughter cells. It should be noted that errors in chromosomal distribution during mitosis are common in normal tissues (approximately 1%), so for criteria to be met for CIN an aneuploidic mitosis must occur in at least 20% of divisions[151, 152]. This must be assessed based on an evaluation of a population of cells in reference to a standard population, rather than observations of individual cells without reference. CIN results from mutations affecting several processes: Chromosome segregation defects, typified by anaphase promoting complex/C dysfunction as a result of MAD (Mitotic Arrest Deficient) and BUB (Budding Uninhibited by Benzimidazoles) mutation; telomere dysfunction resulting in a failure of cells to naturally undergo senescence; and loss of DNA damage response characterised by loss of TP53 function[115].

CIN is present in up to 70% of sporadic CRC, although the methods of determining CIN and the subsequent differences in classifying CIN has led to some discrepancy across the body of literature, although CIN is generally determined as a binary feature of tumours (CIN-positive or CIN-negative)[153, 154]. However, the cellular processes that underlie CIN are not mutually exclusive of those that drive MSI and CIMP, and thus a proportion (up to 25%) of CRC will

demonstrate an overlapping picture of CIN and MSI[155]. CIN-positivity is associated with a worse outcome in CRC; meta-analysis demonstrating higher hazard-ratios for later stage and decreased progression-free survival for CIN-positive tumours[156]. The specific cellular pathways that lead to colorectal carcinogenesis are shared between the genomic aberrations; dysfunction in EGFR, BRAF, and KRAS are again implicated as the victims of CIN within the "mutator phenotype" population of cells that have developed intrinsic genomic instability[157]. The specific role of APC mutations in CIN is also significant; as APC is associated with microtubule and centrosome function in cytoskeletal regulation, and thus mitotic dysfunction, which itself leads to CIN[158, 159].

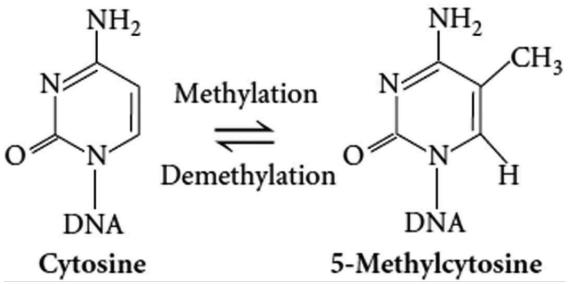
1.3.6 DNA Methylation & CpG Island Methylator Phenotype

DNA hypermethylation and the CpG island methylator phenotype (CIMP) are another significant contributor to the genomic pathogenesis of colorectal cancer and is a key epigenetic mechanism for altering gene expression. As previously discussed, CIN is present in approximately 70% of CRC and MSI-H in 15%, with an overlap of the two accounting for 25%. There are however a significant proportion of sporadic tumours that do not demonstrate either CIN or MSI, and of the tumours where CIN or MSI is demonstrated many do not have the classical Vogelstein mutations in APC, KRAS, and TP53[160, 161]. The third genomic mechanism underlying CRC carcinogenesis is thought to be abnormal DNA methylation, and in particular CIMP.

DNA methylation refers to the addition of a methyl group to the 5-position of cytosine in DNA by DNA methyltransferase (DNMT), to produce 5-methylcytosine (**Figure 1.4**)[162, 163]. Methylation is an active process and is performed by three DNMTs; DNMT1, DMNT3A, and DNMT3B, which are located on chromosomes 19, 2, and 20, respectively. The DNMT family are structurally similar, although 3A and 3B have a slightly lower molecular weight of 100-101kDa, whilst DNMT1 is 183 kDa. Each shows a maximum efficiency for methylation of CpG dinucleotides, and especially islands that are hemi-methylated, although DNMT1 is the most efficient and acts as the primary *de novo* DNA methylator following DNA replication in cell division[164]. Each uses S-adenosyl methionine (SAM) as a methyl donor. An RNA methyltransferase (tRNA aspartic acid methyltransferase 1 – TRDMT1) performs a similar role for RNA.

The most frequent location for methylation of cytosine within DNA is at the CpG dinucleotide pairs that occur sporadically throughout the genome, of which 80-90% are normally methylated in humans. Beyond sporadic CpG pairs, dinucleotide CpG islands (repetition motifs of greater than 200bp) also exist throughout the DNA (1-2% of total) but are not methylated in healthy cells. These CpG islands are often found in the promotor regions of ubiquitously expressed proteins and are thought to facilitate efficient transcription. When these regions become (pathologically) methylated, especially in the context of MSI, they are referred to as hypermethylated CpG islands and affected cells are said to express CIMP, although this process also occurs with ageing and does not always indicate carcinogenesis[165]. Hypermethylation usually results in silencing of the downstream gene.

Figure 1.5 Methylation of the 5-position of cytosine by DNMT to form 5-methylcytosine (from Saini, et.al.)[163].



Both hypo- and hypermethylation have a role in CRC carcinogenesis, although hypermethylation is understood to be the predominant process. Global demethylation has been observed in CRC in the context of specific locus hypermethylation and in association with chromosomal instability, and thus a concept of methylation-redistribution has been proposed, although the hypothesis is contentious[166-169]. The mechanisms that underlie hypermethylation gene silencing are not well understood, although interactions between aberrantly hypermethylated DNA and factors that inhibit transcription such as histone deacetylases and polycomb proteins are thought to dominate, as well as inhibiting pro-transcriptomic agents such as RNA polymerase II[170]. These effects may be mediated by a relatively newly characterised group of proteins that express a common domain called the methyl-CpG-binding domain (MBD), which acts to facilitate inhibitory chromatin restructuring by recruitment of histone deacetylases[171]. Irrespective of the mechanism, it is accepted that the hypermethylation of CpG islands in the promotor regions of a number of genes significantly associated with

CRC, including APC, p16INK4a, and TIMP3, is a genuine epigenetic event that contributes to carcinogenesis by gene silencing[172-175].

Unfortunately, there is no standard definition of CIMP, even within studies focusing on CRC. Early attempts focused on assessing panels of gene promotor regions that were chosen by candidate gene analysis, leading to the 'classic' panel of five loci as described by Park, and including a number of *methylated in tumours* (MINT) sites[176, 177]. An alternative panel was later proposed by Weisenberger based on cluster analysis, and a further panel by Ogino which discriminated between high- and low-level methylation[178-180]. As techniques have evolved further panels were proposed, although whole genome analysis has facilitated a wider understanding of the methylation status of CRC, and techniques in the field are still evolving[181]. **Table 1.2** summarises previously proposed panels for assessing CIMP, with a brief note on their attributes and contribution.

Table 1.2. Summary of CIMP panels and their differing attributes (adapted from Curtin)[165].

Study	CIMP Panel Markers	Note
Toyota, *et.al.* [177]	*CDKN2A (p16)*, MINT1, MINT2, MINT12, MINT17, MINT25, MINT27, MINT31, *MLH1, THBS1.*	Pioneering work to identify markers that distinguish CIMP from age-related methylation.
Park, *et.al.* [176]	*CDKN2A*, MINT1, MINT2, MINT31, *MLH1.*	"Classic" or traditional panel.
Weisenberger, *et.al.* [178]	*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1.*	"New" panel based on stepwise screen of 195 markers.
Ogino, *et.al.* [162]	*CACNA1G, CDKN2A, CRABP1, MLH1, NEUROG1.*	Selected markers to distinguish high-level from low-level methylation.
Shen, *et.al.* [182]	CIMP1: MINT1, *MLH1, RIZ1, TIMP3, BRAF* mutation; CIMP2: MINT2, MINT27, MINT31, Megalin, *KRAS* mutation.	Examined 27 CpG sites, proposed optimal epigenetic and genetic markers to identify CIMP1, CIMP2, or CIMP-.
Tanaka, *et.al.* [183]	*CACNA1G, CDKN2A, CHFR, CRABP1, HIC1, IGF2, IGFBP3, MGMT*, MINT1, MINT31, *NEUROG1, p14, RUNX3, SOCS1, WRN.*	Correlation structures of markers and CIMP differ by *KRAS* and *BRAF* status.
Ang, *et.al.* [184]	202 CpG sites differentially methylated between tumour and normal.	Comprehensive DNA methylation profiling in 807 cancer genes.
Kaneda & Yagi [185]	Group 1: *IGF2, LOX*, MINT1, MINT2, MINT31, *MLH1, RUNX3, SOCS1;* Group 2: *ADAMTS1, DUSP26, EDIL3, ELMO1, FBN2, HAND1, IGFBP3, NEUROG1, RASSF2, STOX2, THBD, UCHL1.*	Comprehensive DNA epigenotyping of genome wide regions identified two groups (high and intermediate to low methylation).

Of the studies examining methylation panels Kaneda and Yagi is the most exhaustive, as a whole-genome approach to assessing CIMP status was undertaken by employing a two-way unsupervised hierarchical clustering method and quantitative methylation techniques[185]. This study clearly demonstrated that CIMP status was clustered into three distinct groups (DNA methylation epigenotypes); CIMP-High(-H), CIMP-Intermediate(-I), and CIMP-Low(-L), with grouped methylation markers determining the classification of each epigenotype[186]. The CIMP-H group was found to correlate strongly with BRAF mutation and MSI-high, and CIMP-I with KRAS mutation, but that classification into each of the epigenotypes was independent of mutational analysis. Of the three sub-classifications, CIMP-I with KRAS mutation demonstrated the poorest prognosis. The loci used for the Kaneda and Yagi classification are outlined in **Table 1.3**, divided into those strongly associated with CIMP-H (Group1) and those with CIMP-I and -L (Group 2). The epigenotyping system is described below.

- CIMP-High if ≥ 2/3 of Group 1 markers are methylated, irrespective of Group 2 markers
- CIMP-Intermediate if 1/3 Group 1 markers and ≥3/5 Group 2 markers is methylated
- CIMP-Low if ≤1 Group 1 markers is methylated and ≤2 Group 2 markers is methylated

1.3.7 Other Epigenetic Factors

Cytosine methylation is only one epigenetic factor influencing gene expression and physiological and pathological phenotype. Another key epigenetic process governing gene expression is histone modification by protein methylation, phosphorylation, or other covalent processes; thus effecting chromatin structure[187, 188]. There is increasing evidence that histone modification may be important in the pathogenesis of colorectal cancer, and several histone-methylation enzymes (acetyltransferases and demethylases) have been associated with oncogenic or tumour-suppressor roles[189]. As the process of histone modification is dynamic and modifiable it presents an opportunity for therapeutic intervention, although this avenue of therapeutics is in its infancy[190, 191].

Table 1.3 Group 1 and 2 methylation markers for Kaneda and Yagi epigenotype classification.

	Gene	Alias	Location	Function
Group 1	SOCS1	SSI1, CISH1	16p13	STAT induced STAT-inhibitor (suppressor of cytokine signalling – SOCS): negative feedback loop signalling in inflammatory cytokine pathways.
	MINT1	APBA-1, X11, IN10	9q21	Member of the X11 protein family: believed to be involved in signal transduction pathways.
	hMLH	COCA1, HNPCC2	3p22	DNA MMR gene. Part of the MutL-alpha complex. Strong association with HNPCC.
Group 2	NEUROG-1	NEUROD 3, NGN-1	5q31	Interacts with CREB-binding protein as a transcription activating co-factor. Protein complex demonstrates histone acetyltransferase activity.
	THBD	THRM, CD141	20p11	Endothelial cell surface membrane protein. Co-factor for thrombin activation.
	HAND1	EHand, Things1, Hxt	5q33	Basic loop-helix-loop transcription protein found in myocardium and associated with vasculogenesis.
	ADAMTS1	METH1	21q21	Multimodular proteins demonstrating a variety of active domains including metalloproteinase activity, disintegrin-like domain, and thrombospontin type 1 motif. Inhibitor of tissue remodelling and anti-angiogenesis.
	IGFBP3	IBP-3, BP-3	7p12	One of six insulin-like growth-factor binding proteins; acts to transport IGFs and modulate interaction at cell surface and at nucleus. High circulating levels associated with worse outcomes in some cancers.

1.3.8 Consensus Molecular Subtyping in Colorectal Cancer

The process of carcinogenesis is now accepted to be an imbalance between the cell-cycle control mechanism and the development of mutations. Evolving from the Vogelstein model, three overlapping genomic and epigenomic pathways are implicated in genomic instability and the carcinogenesis process; chromosomal instability (CIN), microsatellite instability (MSI), and an epigenetic mechanism resulting from DNA hypermethylation called CpG island methylator phenotype (CIMP)[161]. The relationship between these mechanisms and the influence of the tumour inflammatory and immunological microenvironment is complex; and as a greater understanding of the transcriptomic pathways that influence the cancer cell phenotype is developed, the true complexity of CRC tumorigenesis is emerging[118].

One of the earlier molecular classifications for CRC that incorporated methylation status, as well as MSI, CIN, and histopathological characteristics was the Jass Classification[161]. This system separated CRC into five categories that demonstrate a degree of overlap; broadly dividing the serrated from the adenomatous pathways, and splitting the total population 60%-40% between CIMP-negative and CIMP-positive, and CIMP-positive further into 20% CIMP-H and 20% CIMP-L. Unfortunately, subsequent studies aiming to stratify cohorts of patients with CRC based on Jass' classification and then to examine if there is a prognostic significance to the Jass phenotypes have failed both to adequately stratify patients based on the classification and have not demonstrated any significant prognostic implications. The proportions of cancers defined by CIMP status in subsequent studies has also been variable, with Zlobec identifying an approximately 7%, 43%, and 50% split between CIMP-H, -I, and -L, and 41% of tumours not being able to be classified according to the Jass Classification[192]. What was important about this early work, however, was that it introduced the concepts of different molecular pathways and classifications to CRC and provided a framework for their investigation[161, 193].

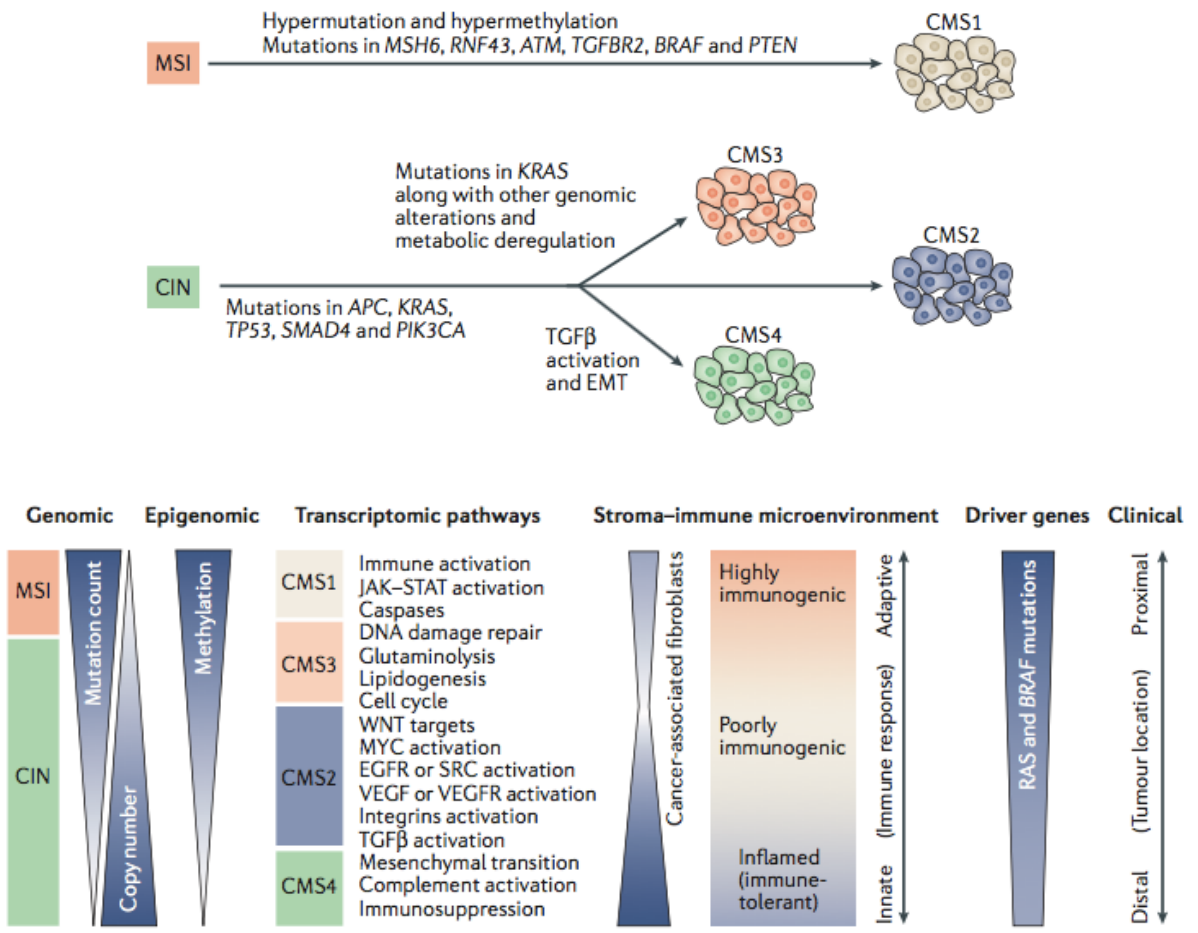
Subsequent studies, many of which were based on whole-genome sequencing and global methylation characterisation, proposed various molecular classifications for CRC and attempted to validate them against large clinical and pathological datasets (**Table 1.4**)[194-201]. Most of these systems have subsequently been abandoned due to failures in external validation, although components of each have been incorporated into and/or informed the subsequent consensus position on molecular subtyping in CRC.

Table 1.4 Molecular & Phenotypical Subtyping in Colorectal Cancer

Study	Classifications	Primary Differentiators	Note
Jass, *et.al.*[161] 2007	Types I - V	CIMP-H, -L, -Nil; MSS/MSI-L, MSI-H	Types I & II associated with serrated pathway, IV & V with Vogelstein, III with either
Perez-Villamil, *et.al.*[197] 2012	Clusters 1 - 4	MSS, MSI; Histology (mucinous); % stromal content; BRAF V600E	Emphasis on the role of the tumour microenvironment to classification and "malignant power"
Schlicker, *et.al.*[200] 2012	Types 1 & 2: subdivided into 1.1, 1.2, 1.3, 2.1, & 2.2	EMT (mesenchymal vs epithelial); MSI, MSS; Pathway upregulation	Demonstrated stratification across 74 immortalised cell lines and differentiation in response to *in vitro* therapies
Budinska, *et.al.*[194] 2013	Types I – V	MSS, MSI; BRAF V600E; p53; KRAS	Division into typically crypt-like (upper & lower), CIMP, mesenchymal, or mixed expressional clusters
De Sousa, *et.al.*[195] 2013	Types 1 – 3	CIN+ve, -ve; MSS, MSI; CIMP-H, -L	Type 3 (MSS CIMP-H) associated with sessile-serrated pathway
Marisa, *et.al.*[196] 2013	Subtypes C1 – C6	Wnt; MSS, MSI; CIN+ve, -ve; KRAS	Subtypes C4 (stem cell-like) & C6 associated with worse relapse-free survival
Sadanandam, *et.al.*[199] 2013	Subtypes 1 - 6	Wnt; Crypt location; MUC2 & TFF3; "Inflammatory"	Subtypes reflect distinct normal crypt cell types and exhibit "stemness". Response to chemotherapy differs between subtypes
Roepman, *et.al.*[198] 2014	Types A, B, & C	BRAF V600E; EMT expression; MSS, MSI	Subtypes may predict prognosis and response to chemotherapy
Guinney, *et.al.*[201] 2015	CMS 1 – 4 [Consensus Molecular Subtype]	Consensus methodology of previous molecular & phenotypic studies	Four streams of subtyping with distinct phenotypic and molecular characteristics.

Current consensus suggests four identifiable molecular subtypes (CMS1-4) based on transcriptomic analysis, the first of which is based upon a high degree of underlying MSI and hypermethylation (including CIMP), as opposed to CIN (**Figure 1.5**)[118, 201, 202]. The further three pathways are based on chromosomal instability with APC dysfunction and the Vogelstein model of compound genetic insult, but subsequent canonical divergence between phenotypes displaying divergent metabolic or inflammatory phenotypes. This collaborative study, based on the large-scale pooling of cohorts and bioinformatic methodologies, has helped characterised CRCs and framed the ongoing discourse in precision medicine as it will apply to the disease. Although not clinically validated (except for KRAS wild-type anti-EGFR therapies), predictive biomarkers are emerging that may in future help guide therapies to facilitate treatment based on individual tumour biology; resulting in less wasteful and more effective care.

Figure 1.6 Consensus Molecular Subtypes 1 - 4 of CRC (adapted from Dienstmann[118]).



1.4 General Aims of this Thesis

Previous work has demonstrated that extramural vascular invasion (EMVI) has an inverse relationship to KRAS mutation, indicating a CIN dominated pathway in this tumour phenotype more typical of the classical adenoma-to-carcinoma pathway often found the distal colon and rectum[203]. MSI-high tumours on the other hand have been associated with serrated polyps and a high prevalence of BRAF mutations in the proximal colon, although a significant percentage affects the rectum and may represent a pathologically worse molecular subtype[125, 147-149]. There is however no current biomarker available to accurately predict the presence of EMVI, although it is suspected that MSI hypermutation and hypermethylation may be the predominating tumourigenic processes in this phenotype.

This thesis will investigate the relationship between methylation and EMVI in rectal cancers, its implications for prognosis, and seek to illuminate the biological processes that underlie any relationship. Each chapter will set out its own objectives, methodologies, results, and conclusions; although the body of work as a whole should constitute a unified enquiry based on this overarching aim. Three principle methodologies will be employed throughout this investigation; assessment of the methylation status of resected rectal cancers and the correlation to prospectively collected clinical and histopathological datasets; *in vitro* studies of methylation in colorectal cancer tissue models, the manipulation of methylation in such models, and biological consequence; and a mechanistic analysis of both tissue models and resected rectal cancer specimens to elucidate the cellular processes that may underlying observed effects.

Chapter 2

General Methodology

2.1 Overarching Considerations & Ethics

2.1.1 Patient Selection

The primary aim of this research was to investigate the relationships between DNA methylation, the pathophysiology of rectal adenocarcinoma (especially extramural vascular invasion), and the mechanisms that underlie any relationship. The key to linking cellular processes with clinical outcomes lies in the dual analysis of DNA extracted from patient tissue samples and the corresponding clinicopathological data. All patients included in this study were drawn from a prospectively maintained database of all patients undergoing treatment for rectal cancer at a single centre (Abertawe Bro Morgannwg University Health Board (ABMU) Singleton & Morriston Hospitals – Swansea Colorectal MDT). Although primarily a local MDT, the Swansea Colorectal MDT has a supra-regional practice in the management of advanced pelvic malignancy requiring sub-specialised surgical and oncological expertise, and therefore captures a greater-than-normal frequency of advanced rectal cancers undergoing treatment with curative intent. Each of the patients included in this study have had surgical treatment for rectal cancer with curative intent, but patients receiving neoadjuvant therapy (nCRT) have been excluded.

Patients who have received neoadjuvant therapy have been excluded due to the likelihood that DNA extracted from tumours previously exposed to nCRT will have undergone epi-/genetic alterations that will serve as confounding factors in analysis. Currently, there is no substantive evidence to associate methylation status with response to nCRT, although there is some equivocation between single/multi locus and whole-epigenome studies, and neoadjuvant demethylation is being explored as a therapeutic augmentation to traditional nCRT in other organ systems[148, 204, 205]. Further exclusion criteria were patients with inflammatory bowel disease, known or suspected high-risk of familial cancer syndromes, recurrent cancers, non-adenocarcinoma tumour types (melanoma, squamous, GIST, etc), and tumours identified as rectosigmoid and beyond 15cm from the anal verge. All patients received pre-operative diagnosis consistent with the prevailing guidelines (most recent; ACPGBI 2017[206]), and

retrieved specimens were retrieved from Singleton Hospital Pathology Department and screened by consultant histopathologist for quality and appropriate tumour representation in accordance with the validated Royal College of Pathologists colorectal cancer minimum reporting datasheet[207].

2.1.2 Database Maintenance & Anonymisation

A prospective database of all patients undergoing treatment at the Swansea Colorectal MDT was maintained throughout and prior to the investigation. Data was primarily extracted directly from the MDT meetings themselves by use of a standard data proforma and real-time data input to the database but was also cross-referenced against a number of clinical and public health resources. Basic demographic information was captured from patient records presented at MDT, and radiological, histopathological, and further clinical data was harvested from respective hospital digitised services. Patient deaths were cross-checked against the NHS Wales Informatics Service (Myrddin), and cancer specific outcomes against the Cancer Information Network System Cymru (CaNSIC) database. Following identification of patients that met inclusion criteria and extraction of raw data from the database, investigators were blinded during the experimental phases of the data collection by use of a dual-labelling methodology (random number allocation) so that no patient-identifiable data was present during laboratory analysis. The two datasets were recombined for dual analysis. All data was stored in accordance with processes set out in the NIHR international Good Clinical Practice (GCP) framework[208].

2.1.3 Ethics

Ethical approval was granted by South West Wales REC (Project Ref No.:11/WA/0256), and sponsored by the R&D Department, Abertawe Bro Morgannwg University LHB, Morriston Hospital, Swansea. Consent was not required in accordance with the Human Tissue Act (2004). All data was handled in accordance with the Data Protection Act (1988 & 2018) and GCP guidelines, and all investigators held current GCP certification[208].

2.2 Materials & General Methods

2.2.1 Laboratory Equipment, Consumables, & Reagents

Throughout this research a great variety of laboratory equipment and consumables was employed during specific processes, each of which will be described in detail in the methods section of each chapter. Described here are the materials and general methods that are common to multiple sections of the research and which will not be described in detail again later. Where these consumables constitute a bought-in kit they will be listed in section **2.2.6**, but thereafter be referred to by their kit name, except where kit modification or process optimisation has taken place. The exceptions to the above are listed below (**Table 2.1**). Items of occasional use are not listed. A laboratory safety folder was maintained throughout.

Table 2.1. Laboratory Equipment & Consumables

Item	**Supplier**
100bp DNA Ladder	Promega, USA
2-well Tissue Culture / Wound Healing Inserts (500µm)	Ibidi GmbH, Germany
30% Acrylamide-1 Bis Sol^{n} (37.5:1)	Sigma-Aldrich (Merck), USA
Albumin (bovine) Serum	Sigma-Aldrich (Merck), USA
Ammonium Persulfate	Sigma-Aldrich (Merck), USA
BICELL Cellular Cryopreservation Vessel	NIHON FREEZER, Japan
Borate	Fisher Scientific, USA
Centrifuges (various)	Various
Corning® Matrigel® Invasion Chamber (6-well plate, 8.0 micron pore)	Corning (Merck), USA
Cryovial 1.8ml	SPL Life Sciences, USA
EpiTect® PCR Control DNA	QIAGEN, Germany
Ethanol	Fisher Scientific, USA
Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific, USA
Interlukin-6 (IL-6) Human (recombinant)	Sigma-Aldrich (Merck), USA
L-Glutamine	Fisher Scientific, USA
Glassware, including microscope slides and cover slips (reusable & single use)	Various
GoTaq® Hot Start Green Master Mix	Promega, USA
Grant Dry Block Heater (variable temperature)	Fisher Scientific, USA
Falcon Tubes 15ml & 50ml (Collar / Conical)	Fisher Scientific, USA
Freezers: -20°C & -80°C	Various & New Brunswick Scientific
Fume Hood	Clean Air, Ltd
Horse Serum	Gibco (Fisher Scientific), USA

Ice Machine (crushed)	Hoshizaki, Japan
Isopropanol	Fisher Scientific, USA
L-Glutamine	Gibco (Fisher Scientific), USA
Light Microscopes x5, x10, x20, x40 (+/- oil)	Various
Methanol	Fisher Scientific, USA
Microcentrifuge Tubes	Eppendorf, Germany
Milli-Q Water Purification System for Type 1 Ultrapure Water	Millipore (Merck), USA
Multi-well Cell Culture Plates (6, 12, 24)	Corning (Merck), USA
Nuclease-Free Water (Ambion)	Sigma-Aldrich (Merck), USA
Paraformaldehyde	Sigma-Aldrich (Merck), USA
PCR Plates (96 well)	Bio-Rad, USA
PCR Biofilm	Bio-Rad, USA
pH Meter	Mettler Toledo, USA
pH Buffers for Calibration	Sigma-Aldrich (Merck), USA
Phosphate Buffered Saline (PBS)	Gibco (Fisher Scientific), USA
Pipette Tips (Tip One System) – various volumes	StarLAB, UK
Pipette Tips (Aspiration) – various volumes	Corning (Merck), USA
Scalpels (disposable)	Swann-Morton, UK
ScanLaf Tissue Culture Hood (Class 2)	BioGene, Denmark
Silver Nitrate	Sigma-Aldrich (Merck), USA
Sodium Hydroxide	Sigma-Aldrich (Merck), USA
Tetramethylethlenediamene (TEMED)	Sigma-Aldrich (Merck), USA
Thermometers (immersion; digital & liquid)	Various
Thinpipette Focus Fixed Volume 5µl, 50µl, 300µl, 1000µl (+ various single & multi-channel)	Fisher Scientific, USA
Tris (hydroxylmethyl) aminomethane	Sigma-Aldrich (Merck), USA
Trypan Blue Solution (0.4%)	Sigma-Aldrich (Merck), USA
Tissue Culture Flasks $25cm^2$ and $75cm^2$ (vented)	Corning (Merck), USA
Vectashield Mounting Medium with DAPI	Vector Labs (Maravai LS), USA
Vortex	Various
Water Baths (37°C)	Various
Xylene	Fisher Scientific, USA
Z1 Coulter Counter	Beckman Coulter, USA

2.2.2 Programmable Equipment

A variety of programmable laboratory equipment was employed at various stages of this research; including but not limited to automated and real-time PCR equipment, microscopes, and tissue-bloc processors. Each item of equipment, its set-up, programming, and analysis will be discussed in the methods section of the relevant chapter; but are listed in **Table 2.2**.

Table 2.2. Programmable Laboratory Equipment.

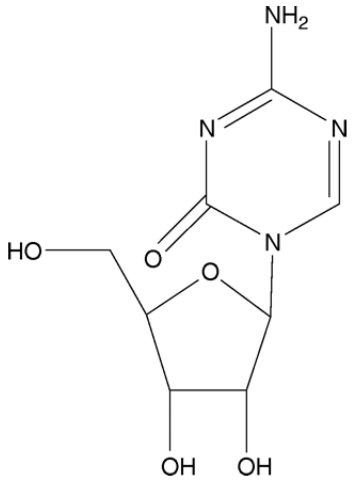
Item & Description	**Supplier**
ChemiDoc XRS+ System - Gel imaging & product analysis	Bio-Rad, USA
etaluma 560 Inverted Live-Cell Microscope - Time-lapse wide-field microscopy	etaluma, USA
IN Cell Analyser 2000 - Automated wide-field cell analysis	GE Life Sciences, USA
IQ-5™ Real-Time PCR System - RT PCR & product analysis system	Bio-Rad, USA
Metafer Automated Slide-Scanning Platform - Automated fluorescence slide-scanning & image capture	MetaSystems Group, Germany & USA
NanoDrop® 1000 UV Vis Spectrophotometer - DNA, RNA, and Protein yield & purity analysis	Labtech (Fisher Scientific), USA
Olympus BX51 Fluorescence Microscope - Multi-filter programmable fluorescence microscopy	Olympus, Japan
T100™ Thermal Cycler - PCR & Microcentrifuge heater block	Bio-Rad, USA
Ventana Benchmark ULTRA - Automated slide processing and IHC	Ventana (Roche), Switzerland

2.2.3 5-Azacytadine

5-azacytidine (AZA) is a pyrimidine ring analogue of the DNA and RNA nucleotide cytidine. After transportation into cells, 5-AZA undergoes de-oxy metabolism to form 5-aza-2′-deoxycytidine, which can then be incorporated into DNA in place of cytidine. Once in C-G dinucleotide formation, DNA methyltransferases (DNMT) bind AZA to catalyse methylation but are inhibited from being released (normally by beta-elimination of the covalent bond at the 5-carbon position) by the presence of a nitrogen[209, 210]. By this mechanism, DNMT activity is inhibited by sequestration of the DNMT enzyme and subsequent failure of de-novo methylation during DNA replication and repair. AZA does not actively demethylate DNA by competitive inhibition of the DNMT's active site, by direct removal of methyl groups from methylated cytosine, or by upregulation of the base excision repair enzyme OGG1 in response to oxidative stress[211]. Due to the necessity of DNA replication to effect demethylation, the cellular and clinical effects of AZA are only evident following multiple cell cycles[212]. DNA demethylation by AZA has been shown to activate previously silent genes, initiate decondensation of chromatin, and induce cellular differentiation; ultimately leading to significant changes in cellular phenotype[213-215].

AZA was supplied by Sigma-Aldrich (now Merck), Germany [A2385]. AZA has a molecular formula of $C_8H_{12}N_4O_2$, a molecular weight of 244.20, and its structure is shown in **Figure 2.1**[216]. AZA was stored at -20°C in its dehydrated form (as shipped), and was dissolved in DMSO/H_2O prior to use, where after it was stored at -80°C for a maximum of one week.

Figure 2.1. Molecular structure of 5-azacytidine.



The effect of demethylation with AZA on DNA are global, and pan-demethylation (hypomethylation) has been demonstrated to contribute to chromosomal instability (CIN) and even to induce malignant transformation *in vivo*; patterns of global demethylation have been observed in a variety of malignant conditions, including T-cell lymphomas (murine model), and human prostate and hepatocellular carcinoma[168, 217, 218]. The consequence of these findings is that AZA may itself be mutagenic due to whole-genome demethylation, and thus some authors have suggested that this may limit its use as a therapeutic agent[168]. The global demethylating effects of AZA at therapeutic doses have however been challenged, with parallel studies examining the demethylating effects of AZA on preserved Alu elements in patients receiving treatment for myelodysplastic disorders indicating that, at therapeutic doses, AZA has only a moderate demethylating effect[169]. The authors of this study speculate that the doses of AZA employed in previous *in vitro* and murine models were not representative of normal physiological conditions (the authors report "extreme modelling"), but they do concede that LINE-1 hypomethylation was observed in their own study of colorectal cancers, although to a less significant degree. The association between CIN and hypomethylation was also investigated in colorectal cell-lines with differing expression of CIN as part of the same investigation, although only a minor, non-statistically significant decrease in methylation between CIN+ and CIN- cell lines were discovered ($p=0.48$)[169]. Other cell-line work has however demonstrated AZA (and its de-oxy derivatives) to be an effective demethylator of oncogenic hypermethylated promotor regions, such as the C/EBPδ tumour suppressor in acute myeloid leukaemia (AML)[219]. In this study, microarray analysis of U937 cells treated with AZA derivatives demonstrated that 274 transcripts were significantly upregulated, including the entire C/EBP family, although C/EBPδ showed a 10-fold expressional increase following demethylation. These findings correlated to the clinical finding that AML patients demonstrated hypermethylation at the C/EBPδ promotor region.

AZA was initially licenced in 2004 by the Federal Drug Administration in the USA for use in all types of myelodysplastic syndrome, including AML, based on one randomised control study and two single-arm studies[212, 220, 221]. Unfortunately, the complete response rate in the treatment arms of these studies was low (16%), although a return to normal blood counts and bone marrow morphology was observed. A partial response was observed in a greater number of patients (19%), but individuals receiving the treatment remained therapy-dependent, with relapse of bone marrow failure following treatment withdrawal. Side-effects were observed in a significant proportion of patients, including 16% who developed severe liver dysfunction,

although minor gastrointestinal and haematological side effects were more common. Despite these poor beginnings, more recent studies employing decitabine (a de-oxy derivative of AZA) showed a greater response rate without increased toxicity or safety concerns, although overall survival was not statistically better between treatment arms, although trend was demonstrated in older (>70yrs) patient groups, especially for *de novo* AML rather than secondary disease[222].

There is currently no role for AZA in the treatment of solid-organ tumours, including colorectal cancer (CRC). Early trials of demethylating agents for a variety of solid-organ tumours, based on the success of therapy for myelodysplastic disorders, were both speculative and disappointing; with very little if any clinical response observed in a mixed cohort of end-stage tumours[223, 224]. When identified as a separate cohort, the results specifically for CRC were especially poor; decitabine monotherapy producing no tumour regression whatsoever in any patient, and a less than 10% response (defined in this study as regression or stable disease) when used in combination therapy. Further studies on patients with end-stage solid-tumour disease also showed no clinical effectiveness when decitabine was combined with a histone deacetylase inhibitor (sodium phenylbutyrate), although toxicity was limited and there were no adverse drug-related events[225].

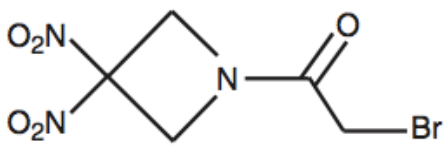
More recently, the potential role of AZA as a chemo-sensitizer in CIMP-high solid tumours has been suggested, based on the observation that CIMP-high has been associated with chemo-resistance in colorectal and other tumours[226, 227]. *In vitro* and xenograft studies have also indicated that demethylation may restore sensitivity to 5-FU therapy in colorectal and other cancer models[228, 229]. Early clinical outcomes have suggested that AZA derivatives may restore partial chemotherapy sensitivity to treatment-refractory non-small cell lung cancers and ovarian cancers, although the numbers in these studies are small and the benefits (in terms of PFS) were marginal[230, 231]. Unfortunately, these results have not been observed in early clinical trials in CRC. In one trial, twenty-six treatment-refractory patients (fourteen CIMP-high) were treated with AZA in addition to standard chemotherapy (CAPOX) to determine if chemo-sensitivity could be restored; no patient responded and there was no correlation between CIMP-status and PFS[232]. A similar study of forty-seven patients with advanced treatment-refractory liver metastasis of colorectal origin also showed no clinical response (by RECIST standards) to combined AZA and entinostat therapy, although tumours demonstrating demethylation had a slight improvement in PFS[233, 234].

2.2.4 RRx-001

RRx-001 (formerly ABDNAZ) is a novel compound produced by modification of the by-products of solid rocket propellant manufacture and has shown some activity suggesting it may have chemotherapeutic or chemo-sensitising properties[235-237]. RRx-001 acts as an alkylating agent, covalently binding nucleophilic targets following liberation of a bromine (the leaving group) in a non-selective and non-reversible manner. This action is similar to that observed with traditional non-selective alkylating agents such as nitrogen mustard (mustard gas) and nitrosoureas, each of which can cause potentially devastating DNA, RNA, and protein damage leading to significant toxicity or death[238]. At a cellular level RRx-001 has been shown to increase reactive nitrogen and oxygen species; resulting in direct DNA damage, increased γH2AX expression reflecting breakage in dsDNA, and apoptosis[237]. The chemotherapeutic mechanisms of RRx-001 are however not well understood, although on *in vivo* modelling, action on the β-cysteinyl residue of haemoglobin and other circulating thiols (glutathione and cysteine) by nucleophilic substitution is believed to underlie the production of circulating nitrogen oxides; thus modulating redux reactions in the circulation and modifying tumour microvasculature through nitrogen dependent pathways. In early trials, RRx-001 did not demonstrate significant "off-target" toxicity, possibly due to the rapid alkylation of haem-components on administration, thus limiting damage to tissues not reached until after the compound has given up its leaving group[235, 239].

RRx-001 was supplied by EpicentRx under experimental licence. RRx-001 is a cyclic nitro compound with molecular formula $C_5H_6BrN_3O_5$, a molecular mass of 268.02, and a molecular structure as shown in **Figure 2.2**. RRx-001 was stored at room temperature in its dehydrated form (as shipped), and was dissolved in DMSO/H_2O prior to use, where after it was stored at -80°C for a maximum of one week.

Figure 2.2. Molecular structure of RRx-001.



RRx-001 was initially investigated as a potential tumour chemo/radio re-sensitiser in the context of end-stage metastatic disease and was trialled against a number of solid organ tumours, including colorectal cancer, and has demonstrated favourable outcomes[236, 237, 240]. The role and mechanisms of small molecule agents in re-establishing tumour sensitivity is not well understood, but some emerging theories suggest epigenetic mechanisms underlie this phenomenon. Supporting this theory are the observations that demethylating agents (including AZA) and histone deacetylating inhibitors have all been shown to have some ability to induce tumour re-sensitisation in previously refractory disease, raising the possibility of "rewriting the epigenetic code" as a means of modulating a tumour's response to therapy[241, 242]. RRx-001 has been demonstrated to cause global DNA demethylation in squamous-cell cancer cell lines *in vitro* (SCC VII), and reduce DNMT1 and DNMT3a expression in a time-and dose-dependent manner[243]. In this study, global DNA demethylation was comparable to that observed with equivalent doses of AZA, although at an individual gene level there was a high degree of variance in the effects; some loci demonstrating hyper-and some hypo-methylation.

In clinical trials RRx-001 has been shown to be biologically active at safe doses in target populations (those with advanced metastatic solid organ tumours), and to be a potent generator of reactive nitrogen and oxygen species *in vivo*[244, 245]. A number of case reports and Phase I trials against metastatic or end-stage Non-Small and Small Cell Lung Cancers, squamous tumours, prostate cancers, and melanomas have indicated that RRx-001 may have beneficial effects when combined with previously exhausted chemo- or radio-therapeutic strategies[246-251]. A current Phase III trial is examining the role of RRx-001 in platinum-treatment-refractory Small Cell Cancer (lung & non-lung), with progression-free survival of 12 months the primary outcome, estimated to be complete by 2020[252]. RRx-001 has also been shown to have immunomodulating effects in the tumour microenvironment in neuroendocrine tumours, and has demonstrated the ability to enhance tumour-related macrophage polarisation and T-lymphocyte infiltration[253-256]. Further studies have suggested a link between RRx-001 induced demethylation and activation of interferon responsive genes in colorectal cell lines (HCT 116); inhibiting tumour cell growth and activating cellular anti-viral defence by "viral mimicry"[257].

Despite a growing body of research into the cellular and tumour-related effects of RRx-001, the exact mechanisms of its action are not well understood, and it is likely that it acts via number of different mechanisms that contribute to its effect. There is however reliable evidence to suggest that at least some of these effects are mediated by a demethylating action.

2.2.5 Primers

Methylation specific PCR primers were selected, designed, and optimised by myself and other members of the research group based on previous enquiry[204, 258]. Metalloproteinase pathway cDNA primers were selected from open-source references by the author and optimised in-house. Each primer was ordered through Sigma-Aldrich; and is summarised in **Table 2.3**. PCR conditions will be described in relevant chapters.

Table 2.3 Primers. U, Un-Methylated; M, Methylated; FWD, Forward; REV, Reverse; MW, Molecular Weight; DM, Dimerization; 2nd, Secondary (VW – very weak; W – Weak; M – Moderate; S – Strong).

Name	MW	DM	2nd	Sequence 5'-3'
hMLH U FWD	7477	No	None	AGAGTGGATAGTGATTTTTAATGT
hMLH U REV	7534	No	None	ACTCTATAAATTACTAAATCTCTTC
MINT1 U FWD	8603	No	None	AATTTTTTTATATATATTTTTGAAGTGT
MINT1 U REV	6603	No	None	AACAAAAAACCTCAACCCCACA
SOCS1 U FWD	9332	No	None	TTTTTTGGTGTTGTTTGGAGGTTGGATTTT
SOCS1 U REV	10681	No	None	AAAACAAAACAATAAACTAAAACACTACAAAACCA
HAND1 U FWD	6233	No	None	AATAGTTTAGGGTGTTGGTT
HAND1 U REV	5700	No	None	AAATTTTACACTCAACCCA
ADAMTS1 U FWD	7831	No	None	GTGAGTAATATTGTAGTTAAGGTGG
ADAMTS1 U REV	7582	No	None	AAAACAAAAAAACACTCTAAAACTCC
NEUROG U FWD	7482	No	None	TTGTTGGTTAATTGGTGGTGTTGT
NEUROG U REV	7459	No	VW	CATTACCTCAACCACTAATCACCCA
IGFBP3 U FWD	7704	No	None	TTATTTTGGTTTTTATATAGTGGTT
IGFBP3 U REV	7573	No	None	AACAAAAAACAACTAATCCTCAACA
THBD U FWD	7407	No	None	ATGTGTTTGTTTTTATTTGGTGTT
THBD U REV	6618	No	None	CATAACTAACCAAAAACCCACA
hMLH M FWD	5514	No	M	GATAGCGATTTTTAACGC
hMLH M REV	7261	No	None	TCTATAAATTACTAAATCTCTTCG
MINT1 M FWD	7939	No	None	AATTTTTTTATATATATTTTCGAAGC
MINT1 M REV	5407	Yes	None	AAAAACCTCAACCCCGCG
SOCS1 M FWD	5881	No	None	TTGTTCGGAGGTCGGTTT
SOCS1 M REV	6088	No	None	ACTAAAACGCTACGAAACCG
HAND1 M FWD	6203	No	None	AATAGTTTAGGGCGTTGGTC
HAND1 M REV	5419	No	None	AATTTTACGCTCAACCCG
ADAMTS1 M FWD	7891	No	None	GTGAGTAATATCGTAGTTAAGGCGG

ADAMTS1 M REV	7629	No	None	CTAAAACAAAAAACGCTCTAAAACG
NEUROG1 M FWD	6492	No	None	AATTTATGTTCGCGGGAGGTC
NEUROG1 M REV	6024	No	None	ACCAACTTAACCCGAACCGA
IGFBP3 M FWD	6737	No	None	TTTCGGTTTTTATATAGCGGTC
IGFBP3 M REV	6689	No	None	AAAAAACGACTAATCCTCAACG
THBD M FWD	6385	No	None	CGTTCGTTTTTATTCGGCGTC
THBD M REV	5678	No	None	GCCAAACCCCATCTCATCG
MMP2 cDNA FWD	6926	No	None	TTTCCATTCCGCTTCCAGGGCAC
MMP2 cDNA REV	7473	No	None	TCGCACACCACATCTTTCCGTCACT
MMP9 cDNA FWD	5979	No	None	CCTGCCAGTTTCCATTCATC
MMP9 cDNA REV	6019	No	None	GCCATTCACGTCGTCCTTAT
MMP11 cDNA FWD	6133	No	W	GGGGATGTCCACTTCGACTA
MMP11 cDNA REV	6262	No	None	CAGTGGGTAGCGAAAGGTGT
MMP13 cDNA FWD	6057	No	None	AACATCCAAAAACGCCAGAC
MMP13 cDNA REV	6109	No	None	GGAAGTTCTGGCCAAAATGA
TIMP2 cDNA FWD	7135	No	W	GGCGTTTTGCAATGCAGATGTAG
TIMP2 cDNA REV	6975	No	W	CACAGGAGCCGTCACTTCTCTTG
TIMP4 cDNA FWD	6087	No	W	AGACCTCACAGGCTCAGTCG
TIMP4 cDNA REV	6029	No	W	CATTCCTGCCAGTCAGCCTG
bACT FWD	5492	No	None	GATGGCCACGGCTGCTTC
bACT REV	5550	No	S	TGCCTCAGGGCAGCGGAA

2.2.6 Kits

To facilitate the reproducibility and maintenance of standards across replications of experimental cycles, and to provide literature-referenced and evidenced methodologies, a number of bought-in experimental kits were employed. Kits reduced the necessity for in-house method design and development and were also a convenience. Deviations or optimisation of kit protocols will be discussed in each relevant chapter methodology. Kits are listed in **Table 2.3**.

Table 2.3 Kits

Name	**Use**	**Supplier**
DNeasy® Blood & Tissue Kit	DNA extraction & purification.	QIAGEN, Germany
EpiQuik™ DNMT Activity/ Inhibition Assay Ultra Kit (colourimetric)	Measurement of DNMT activity or inhibition based on nuclear extracts.	Epigenetek, USA
EpiQuik™ Nuclear Extraction Kit	Nuclear protein extraction.	Epigenetek, USA
Imprint® DNA Modification Kit	One-step DNA bisulfite conversion & post-modification clean up.	Sigma-Aldrich (Merck), USA
MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (colorimetric)	Detection of global DNA methylation.	Epigenetek, USA
QuantiFast® SYBR® Green RT-PCR Kit	Quantitative one-step PCR.	QIAGEN, Germany
QuantiTect® Reverse Transcription Kit	Reverse transcription of RNA to cDNA with gDNA wipe-out	QIAGEN, Germany
RNeasy® Mini Kit	RNA extraction & purification.	QIAGEN, Germany
RT^2 First Strand Kit	cDNA synthesis for RT PCR	QIAGEN, Germany
RT^2 Profiler™ PCR Array – Human Tumour Metastasis	Pathway & process specific quantitative RT PCR	QIAGEN, Germany

2.2.7 Antibodies & Immunofluorescence

Antibodies were employed at two stages during this research; firstly during expressional analysis and assessment of knock-down by indirect immunofluorescence (IIF) in cell culture experiments, and secondly during immunohistochemistry (IHC) of tissue microarrays. Each process will be discussed in detail in the relevant chapter, but antibodies employed are listed in **Table 2.4**. Appropriate horse-radish peroxidase conjugated secondary antibodies were used for Western Blotting.

Table 2.4 Antibodies

	Name	**Supplier**
IIF	MMP-2 Monoclonal Antibody [sc-13594] (mouse, IgG)	Santa Cruz Biotech, USA
	TIMP-2 Monoclonal Antibody [sc-365671] (mouse, IgG)	Santa Cruz Biotech, USA
	TIMP-4 Polyclonal Antibody [PA5-30228] (rabbit, IgG)	Fisher Scientific, USA
IHC	MMP-2 Monoclonal Antibody [EPR1184] (rabbit, IgG)	Abcam, USA
	MMP-9 Polyclonal Antibody [ab38898] (rabbit, IgG)	Abcam, USA
	MMP-11 Polyclonal Antibody [ab119284] (rabbit, IgG)	Abcam, USA
	TIMP-2 Monoclonal Antibody 3A4 [ab1828] (mouse, IgG)	Abcam, USA
	TIMP-4 Polyclonal Antibody [ab58425] (rabbit, IgG)	Abcam, USA
2ndary	Goat Anti-Rabbit IgG H&L Alexa Fluor® 488 [ab150077]	Abcam, USA
	Goat Anti-Mouse IgG H&L Alexa Fluor® 488 [ab_2534060]	Fisher Scientific, USA
	OV HQ Universal Linker + HPR Multimer	Ventana (Roche), Switzerland

2.2.8 Software

Table 2.5 summarises the primary computer software employed during this research (not including benchtop equipment with integrated software – see section **2.2.2**), including plug-ins, but excluding web browsing software and operating systems.

Table 2.5 Software

Name & Edition	Primary Role
Image J (v.1.51)	Image analysis
Image Lab 6.0.1	Gel analysis
Lumaview v.17.11.04	Time-lapse microscopy
Metafer 4 v. 3.8.5 (MSearch)	Slide handling and microscopy
Microsoft Office (Word v.16.12, Excel v.16.12)	Word processing, data handling & analysis
XLStat, Addinsoft (v.2017)	Plug-in statistics package for MS Excel

2.3 Cell Lines & Culture

2.3.1 Cell Lines

2.3.1.1 DLD-1

The primary cell line used as a model for rectal cancer throughout this research is DLD-1, originally sourced from the European Collection of Animal Cell Cultures (ECACC 90102540), and maintained in-house (average passage 7)[259]. DLD-1 is a human colon epithelial cell line derived from an adenocarcinoma in a male patient (also the source of colorectal cell lines HCT-15, HCT-8, and HRT-18)[260]. DLD-1 have a 2n = 46 pseudodiploid karyotype. DLD-1 has an adherent growth mode and is best cultured in RMPI 1640 + 2mM glutamine + 10% foetal bovine serum (FBS). DLD-1 was selected as it is a widely used cell line for the investigation of colorectal cancer *in vitro* and has been used extensively in the proceeding and preparatory studies for this research[204]. DLD-1 demonstrates a high level of microsatellite instability and is CIMP-positive across two epigenomes commonly examined in tumour DNA methylation research, in addition to our own methylation panel, and thus serves as a good model for investigating consensus molecular subtype I tumours. DLD-1 is also CIN-negative and BRAF *wt*, but demonstrates KRAS G13D amino acid substitution relating to underlying mutation[261, 262]. Unfortunately, there is no commercially available rectum-specific human adenocarcinoma cell line.

2.3.1.2 HFL-1

Human foetal lung fibroblasts, HFL-1, were used as positive controls for immunocytochemistry of metalloproteinase expression in knockdown experiments, originally sourced from the European Collection of Animal Cell Cultures (ECACC 89071902), and maintained in-house (average passage 5)[263]. HFL-1 have a 2n = 46 diploid karyotype. HFL-1 has an adherent growth mode and is best cultured in Ham's F12 + 2mM glutamine + 1% NEAA + 10% FBS.

2.3.2 Cryopreservation

Cells were grown to a maximum of 70% confluency and then washed twice with sterile phosphate buffered saline (PBS), lifted with trypsin, and then washed a further two times with PBS by serial centrifugation and re-suspension. Cells were then diluted to a concentration of $1x10^6$ cells ml^{-1}, spun to pellet, and resuspended in FBS and DMSO at a ratio of 9:1 maintaining the starting concentration. Aliquots of 1ml were then transferred to cryovials and frozen for

24 hrs at -80°C in a BICELL Cellular Cryopreservation Vessel. Frozen cryovials were then transferred to liquid nitrogen at -196°C for long-term storage.

2.3.3 Cell Seeding & Maintenance

Standard laboratory personal protective equipment (lab coat and gloves) were employed to maintain personal protection and prevent cross-contamination. Surfaces were washed with 70% ethanol and allowed to air dry. All culture took place in ScanLaf Class II Tissue Culture Hoods. Frozen cells were incubated at 37°C with 5% CO_2 prior to seeding, and all culture media & solutions (PBS, trypsin, etc) were pre-warmed to 37°C. Active, adherent cell lines were detached from their growing environment by incubating in warmed trypsin for 5-10 minutes after washing.

Cell lines were maintained in their growth medium of preference (see section 2.3.1) at a maximum of 70% confluency. Cells were initially seeded at a concentration of $3x10^4$ cell ml^{-1} based on published cell growth rate / doubling times in $75cm^2$ flasks (working volume typically 25mls) and allowed to proliferate. Cells were maintained in flasks for a maximum of four days between splitting, and concentrations were determined using a Beckman Coulter Counter. Cells were removed from maintenance culture and split to experimental flasks (typically $25cm^2$, working volume 5mls) or other culture environments according to protocol; each of which will be discussed in the relevant chapter.

Chapter 3

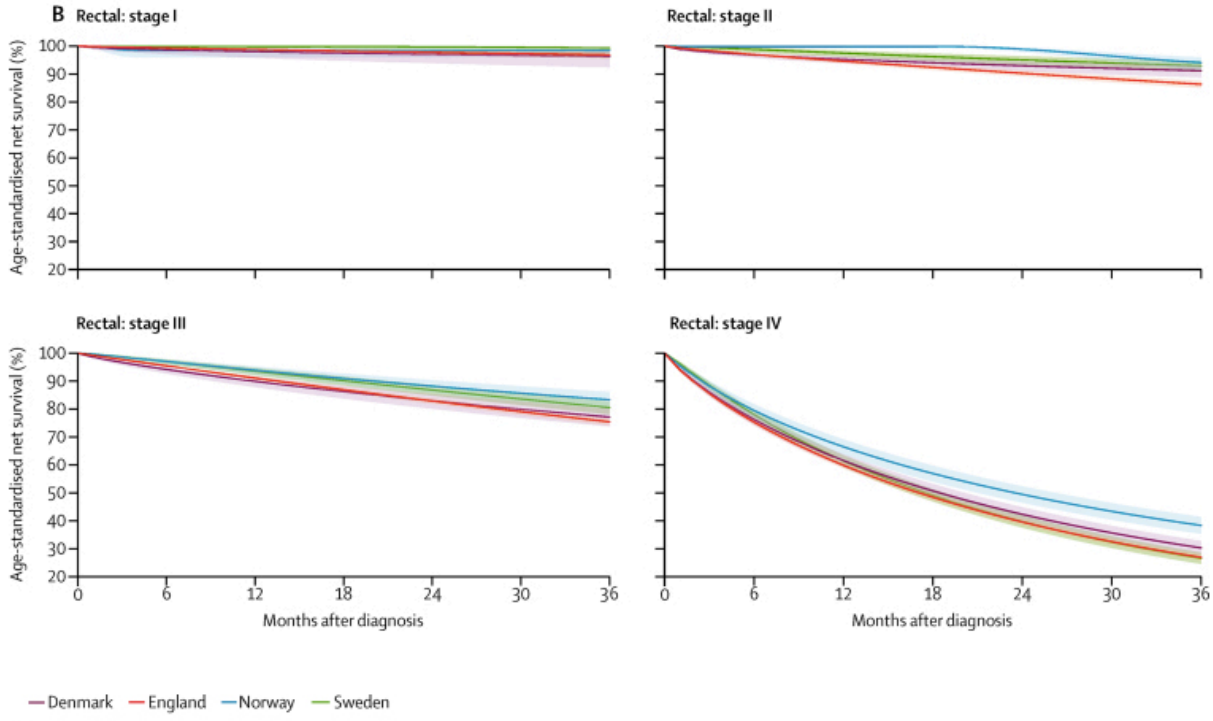
DNA Hypermethylation as a Predictor of Extramural Vascular Invasion (EMVI) in Rectal Cancer.

3.1 Introduction

3.1.1 Epidemiology of Rectal Cancer

Colorectal cancer (CRC) is the third most frequent cancer and the second leading cause of cancer death worldwide[264]. Each year, one million people will develop CRC, and 40-50% will die within five years[265]. Furthermore, rectal and distal sigmoid cancers are known to present at a later stage, and have a poorer prognosis than other colonic cancers[266]. Data from the Surveillance, Epidemiology, and End Results programme (SEER) indicates that between 1995 and 2008 there were 110,607 cases of rectal cancer recorded in the US (estimated population capture 25%), representing approximately 30% of all colorectal cancers[267]. Patients presenting were on average five years younger than those with colonic cancer (median age 67 vs 72 years; p=<0.001) and were more likely to be male (56.8%; p=<0.001). This data is in keeping with the most recent National Bowel Cancer Audit from the UK (NBOCA 2018), where of the 30,541 patients diagnosed with colorectal cancer between April 2016 and March 2017, 8,514 (27.9%) had a rectal cancer, and of whom 53% progressed to major resection and 7% to local excision[268]. A recent European population-based study of 139,457 patients with colorectal cancer from England, Denmark, Norway, and Sweden demonstrated that the pooled (all stages) 3-year survival from rectal cancer in England was actually better than that for colon cancer (69.7% vs 63.9%), but that survival for both cancer locations was worse in England compared to the other Northern European countries[269]. Survival in England was notably worse in stage II and III rectal cancers (and stage IV colon cancer), as demonstrated in **Figure 3.1**, with the number of patients undergoing surgery for rectal cancer lowest in England (59.9%) when compared to other nations (highest: Sweden, 70.8%). A systematic review of surgery for locally advanced or locally recurrent rectal cancer (1016 patients) demonstrated a mean overall survival of 31 months and median 5-year survival of 32%[270].

Figure 3.1 Northern European age-standardised survival for rectal adenocarcinoma diagnosed between 2010 and 2012 (adapted from Benites Majano et.al.)[269].



3.1.2 Extramural Vascular Invasion in Rectal Cancer

Extramural vascular invasion (EMVI) is characterised by the presence of organised tumour in the vessels adjacent to the primary tumour beyond the muscularis propria and has been reported in approximately 25% of resected rectal tumours, although the reported incidence has varied between 17% to 70%[271-273]. Despite its recognition within pathological specimens for some time, defining and recording its presence as a separate entity from other T3 tumours has not been consistent, although the current reporting criteria issued by the Royal College of Pathologists stipulates that it is a mandatory reporting criterum[207, 274, 275]. EMVI has been demonstrated to be an important risk factor for systemic recurrence, local recurrence and death, independent of T stage[272, 273, 276-279]. Additionally, EMVI status influences the need for neoadjuvant chemoradiotherapy and adjuvant chemotherapy, as it has been demonstrated that chemoradiation (CRT) can cause vessel fibrosis in EMVI-positive tumours; which may influence survival outcomes[85, 280].

Although magnetic resonance imaging (MRI) has an undisputed role in defining involvement in the circumferential resection margin (CRM) in rectal cancers, as demonstrated in the MERCURY trials, it's role in assessing EMVI not threatening the CRM is not as well defined[281-284]. The outcome of the MERCURY II trial (2016) demonstrated the diagnostic accuracy of MRI in predicting CRM involvement in low rectal cancers, and in stratifying them into "safe" and "unsafe" groups that would or would not benefit from neoadjuvant radiotherapy and/or sphincter saving surgery[285]. This prospective validation also demonstrated the utility of MRI in assessing "safety" following neoadjuvant therapy, allowing the potential for sphincter-saving operations to be considered for those who had shown disease response.

However, the seminal study of MRI assessed EMVI led by G. Brown in Cardiff in 2003 suggested that there was a weighted agreement of κ=0.64 between pre-operative MRI and histopathology, although the total study population was only 98 patients, of whom 26 had histologically confirmed pEMVI and of these only 18 were visible to the naked eye with standard staining techniques[284]. The study suggested that the relatively poor concordance, even when compared to a naked-eye assessment of histology specimens, was the result of MRI not being sensitive enough to detect mrEMVI in vessels of less than 3mm. Concordance between mrEMVI against pEMVI identified at microscopy would therefore be even less favourable due to a higher denominator. In a subsequent study led by the same author, mrEMVI (as defined by a 0-5 point scoring system, later dichotomised into mrEMVI-positive or -negative), has a sensitivity and specificity of only 68 and 88 percent, respectively, compared to histology[280]. However, despite poor sensitivity and specificity, univariable analysis of 3-year relapse-free survival for each group was highly significant (35% vs 75%, p=<0.001) and corresponded well with outcomes based on histology.

The predictive prognostic significance of MRI detected EMVI has been further evaluated in larger cohort studies, such as that by Bugg in 2014; this study demonstrated that within a population of 788 patients with rectal adenocarcinoma diagnosed between 2007 and 2012, 26.2% had an mrEMVI-positive tumour and of this proportion approximately 25% went on to develop liver metastasis within 1 year, compared to 6.7% of mrEMVI-negative tumours (p=<0.001, RR=3.7)[286]. These findings have been corroborated further by meta-analysis of 1262 patients with rectal cancer (403 mrEMVI-positive), where EMVI was found to be associated with a higher chance of presenting with or developing metastasis (OR=5.68 & OR=3.91, respectively, both p=<0.001)[287].

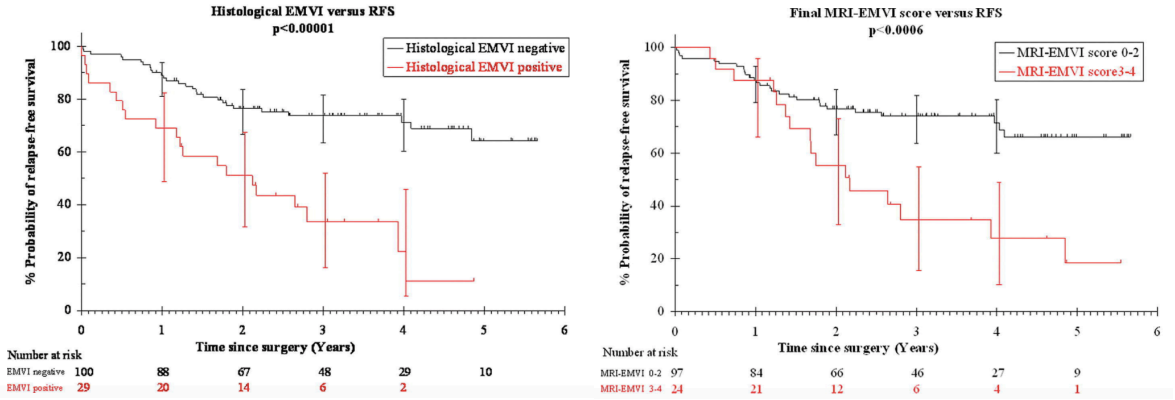
However, the lack of preoperative MRI diagnostic certainty for staging beyond CRM clearance was clearly demonstrated by a large meta-analysis of 21 studies by Al-Sukhni published in 2012[288]. MRI specificity for CRM involvement was 94% (95% CI 88-97) despite a high degree of inter-study heterogeneity, but specificity fell to 75% (95% CI 68-80) and 71% (95% CI 59-81) for T and N stage, respectively.

The presence of mrEMVI is therefore considered an important component of pre-operative staging but is largely limited to its impact on the CRM. The presence or absence of EMVI is included in several international reporting proformas for preoperative radiological disease staging, including the European Society for Medical Oncology guidelines for the diagnosis and management of rectal cancer and the Royal College of Radiologists guidelines for cross-sectional imaging in cancer management[289-292]. mrEMVI does not however constitute a specific item within of the preoperative TNM system advocated by the Union for International Cancer Control (UICC), which does not discriminate between a T3 primary tumour mass and serpiginous and/or distant vascular tumour extension as described by Brown in 2003[280]. Interestingly, the ACPGBI 2017 Guidelines for the Management of Colorectal Cancer (Investigation & Staging section) does not mention mrEMVI in the context of preoperative staging; only that threat to CRM should be assessed by MRI [Recommendation grade B] (see section 2.2.1 of the 2017 guidelines, p12)[206]. Again, this suggests that mrEMVI is regarded as only significant when threatening the CRM and is not regarded as a separate pathological entity within the broader context of T3 disease, although conflictingly, mrEMVI is discussed in the same 2017 guidelines (Multidisciplinary Management section, see section 4.1.2, p28) where it is highlighted as a risk factor for local and distant recurrence independent of T stage (**Figure 3.2**)[293]. However, despite discrepancies in detection and classification, pre-operative mrEMVI has been shown to be a poor prognostic feature irrespective of subsequent management strategy; mrEMVI being associated with a four-fold increase in risk of distant metastasis (52% vs 12%) and reduced 3-year relapse free survival at three years (35% vs 74%)(**Figure 3.3**)[294].

Figure 3.2 Risk of local recurrence following resection of rectal cancer with curative intent based on preoperative MRI findings (adapted from Gollins, et.al.)[293]. Note that EMVI is listed as an independent risk factor for a moderate increased risk of local recurrence.

Risk of pelvic local recurrence	Characteristics of rectal tumours predicted by MRI
Low (resectable)	cT1 or cT2 or cT3a **and** no lymph node involvement
Moderate (resectable)	any cT3b or greater, in which the potential surgical margin is not threatened **or** any suspicious lymph node not threatening the surgical resection margin **or** the presence of extramural vascular invasion
High (borderline resectable or unresectable i.e. threatened or involved CRM)	a threatened (<1 mm) or breached resection margin **or** low tumours encroaching onto the inter-sphincteric plane or with levator involvement

Figure 3.3 EMVI is associated with worse 3-year relapse-free survival (RFS) irrespective of whether it is detected preoperatively (mrEMVI) or at histopathology, irrespective of management strategy (adapted from Smith et.al)[280].



It is clear therefore that the presence of EMVI is a marker of poor prognosis, but it is not clear how this should affect management strategy. Even in the absence of a threatened CRM neoadjuvant short-course radiotherapy (SCPRT) and long-course chemoradiotherapy (LC-CRT) have been shown to reduce local recurrence by approximately 50%, although the absolute benefit is small (5-6%) and the numbers needed to treat are high (17-20), resulting in a high proportion of futile overtreatment. Both SCPRT and LC-CRT have no effect on the risk of systemic recurrence or overall survival. Despite lacklustre benefits, the most recent ACPGBI guidelines suggest that all patients with high-risk CRM clear disease (including mrEMVI positive) should be considered for either SCPRT or LC-CRT to reduce the chance of local recurrence [Recommendation grade A], although as shown by the NBOCA data, clinical practice is highly variable across MDTs[268, 293].

The role for neoadjuvant therapy in locally advanced and CRM-threatened rectal cancer is well evidenced; based on multicentre international randomised-controlled trials (the Dutch trials and UK MRC CR07 trial)[295-297]. Evidence for treatment is irrespective of whether it is EMVI or primary tumour extension that is threatening the CRM, and the primary benefit is in local disease control (LR). The current standard of care in this situation is LC-CRT with a single-agent fluoropyrimidine and radiotherapy, followed by surgery 6-12 weeks after treatment to allow tumour regression; based on large trials demonstrating the superiority of combined chemo- and radiotherapy (EORTC 22921 and FFCD 9203)[79, 80, 99]. Neoadjuvant CRT was further demonstrated to be superior to adjuvant CRT, in terms of LR and reduced toxicity, in the German GAO/ARO/AIO-94 trial, although again, no DFS or OS benefit was evident[78, 298]. Interestingly, long-term outcomes from the German study indicate that perioperative complications, including anastomotic leak and wound complications were associated with poor overall and disease-specific survival (recurrence and metastasis), and that surgical complication was predictive of reduced OS based on Cox regression analysis ($p=0.008$). Short-course protocols were found to be inferior regarding pCR rates (Polish trials), although LR, DFS, and OS were not significantly affected (TROG trial)[299-301].

However, as discussed in **Section 1.2.2**, a significant proportion of patients will undergo a PCR following neoadjuvant CRT, although a further significant proportion will progress during therapy; including the development of EMVI, progression of EMVI, local tumour extension, and development of metastatic disease[72-75]. The response to therapy ultimately dictates surgical strategy, presenting the opportunity for both organ and sphincter preserving techniques, but also for progression and palliation. Currently there is no reliable pre-treatment test to aid in defining which patients will benefit from neoadjuvant therapy for either high-risk CRM-clear or CRM-threatened/involved disease, although biomarkers, including CIMP, are being examined as potential aids to direct therapy[76, 77].

3.1.3 Epigenetic Biomarkers in Colon & Rectal Cancer

3.1.3.1 Introduction

Hypermethylation and hypomethylation in colorectal cancer were first observed by Feinberg and Vogelstein in 1983, occurring at CpG islands and resulting in transcriptomic silencing of tumour suppressor genes and genes involved in DNA mismatch repair[302, 303]. As associations are made between the development of colorectal cancer and epigenetic aberrations there is a potential for epigenetic analysis to help guide our diagnosis and management of colorectal cancer[304]. Gaining an insight into tumour behaviour, including diagnosis, prognosis, and response to therapy by analysing epigenetic changes may potentially improve outcomes if they can effectively guide therapy[305, 306]. Important pathways in cell regulation have already been implicated in the epigenetic pathogenesis of CRC; including those of the Wnt pathway (APC, AXIN2, DKK1, SFRP1, SFRP2, WNT5A), the CDKN2A cell-cycle genes, RAS signalling, and the DNA repair genes MGMT and hMLH1 & hMLH2[307-309]. However, despite epigenetic silencing of classical tumour suppressor genes and dysregulation of other cancer-related pathways having been demonstrated in CRC, epigenetic profiling has only been implemented in guiding personalised therapies in a limited number of clinical scenarios. Specific examples are in the management of some gliomas and prostate cancers, where in the case of gliomas, the hypermethylation-related silencing of O^6-methylguanine-DNA methyltransferase (MGMT) is predictive of response to treatment by alkylating agents, although the beneficial response in the hypermethylated group is eventually lost and all patients relapse[310-312]. The reason for limited implementation may lie in the complexity of the epigenetic factors potentially involved in tumourigenesis; thus epigenome-wide studies such as the BLUEPRINT and NIH Roadmap projects are being performed to map the human DNA methylome to provide reference for investigators seeking disease-specific factors[313-315]. It is hoped that these projects will provide the groundwork for further insight and prove as important for epigenetics as the Human Genome Project was for genetics. Epigenetic techniques do not however have to be considered in parallel to classical genetic methods, and the combination of the two can usefully be applied in screening and other tests, as has already been demonstrated in small studies of stool DNA analysis for adenoma and carcinoma[316]. It is worth noting at this point that much of the research into epigenetic aberrations in lower gastrointestinal cancers fails to distinguish colonic from rectal cancers, and thus much of the literature refers to colorectal cancer in general, although a small sub-population do focus on individual tumour sites. This issue is highlighted by Jia in a meta-analysis of CIMP and its relationship to

outcomes in colorectal cancer, where only 1/30 included papers were specific to rectal cancer[317, 318].

3.1.3.2 Diagnosis

Identifying and validating the consistency of aberrant methylation in CRC opens the possibility that DNA detected in serum or stool may provide a non-invasive means of diagnosing patients with CRC, and even detecting adenomas[319]. Currently there are three commercially available serum tests for colorectal cancer, each of which is based up detecting hypermethylated (m)SEPT9 tumour DNA (Epi proColon® 2.0; ColoVantage®; Real Time mS9®)[320-323]. Each test employs a qualitative PCR methodology to detect the presence of mSEPT9 (normally an unmethylated gene coding a protein involved in pseudopod protrusion and cell migration), but individually reported sensitivities range from approximately 40%-95% and specificities of 80%-90% based on training and test cohorts of 250-1544 patients (typically 1:2 ratio of cancers to controls)[320, 324]. However, a recent meta-analysis by Song identified 25 investigations including 8643 participants (2613 CRC cases) examining the diagnostic accuracy of mSEPT9 serum tests and reports that in the asymptomatic population mSEPT9 is inferior to FIT testing, although it may be superior in the symptomatic group[325]. The diagnostic accuracy described in this analysis was widely variable, with pooled analysis indicating the overall sensitivity for mSEPT9 ranged between 36.4% and 93.4%, dependent on the test algorithm employed and disease stage. The utility of mSEPT9 is further limited by heterogeneity in the assays and test-models employed in binarizing or stratifying at-risk groups, its projected poor cost-effectiveness in population-based screening, and limited utility in detecting adenomas[326]. These findings are corroborated by the American College of Gastroenterology led Multi-Society Taskforce on Colorectal Cancer, who highlight the 48% crude sensitivity for cancer detection of mSEPT9 reported by Church (sensitivity ranged 35%-78%, increasing with disease stage), and thus do not recommend it as a diagnostic or screening test[327, 328].

Beyond mSEPT9, a number of other serum methylation-based diagnostic tests have been trialled, although in a limited manner. A study examining the methylation status of frizzled-related protein 2 gene demonstrated a sensitivity of 67% across serum, blood, and tumour samples, and a serum-based screening methodology identified methylated THBD as a potential biomarker for CRC, although it was only sensitive and specific (75% and 80%, respectively) for stage II and III disease[329, 330]. Compound tests employing multiple methylation markers have also been trialled with a degree of success. In a study of 243 patients, a test model (M-

score) analysing hypermethylation at a set of four genes (APC, MGMT, RASSF2A, and Wif-1) was sensitive (86.5%) and specific (92.1%) for the presence of sporadic colorectal cancer and large adenomas, translating to a positive- and negative-predictive value of 90.6% and 88.8%, respectively (test cut-off 1.6)[331]. A further study based on a candidate-epigene methodology (restriction analysis) and subsequent microarray and RT-PCR analysis identified three hypermethylated markers (TMEFF2, NGFR, and SEPT9) with acceptable test binarization based on circulating DNA analysis between patients with colorectal cancer and controls, although not at a high enough level to provide a utilisable clinical test[332]. Further studies of circulating tumour DNA methylation have proposed other epigenetic targets, although none has yet been translated into an implementable screening tool[333, 334].

Methylation-based stool tests have also been trialled, and currently a methylation analysis of the vimentin gene is under development in the USA, boasting a sensitivity of 83% and specificity of 82% irrespective of tumour location, stage, or patient age[335]. However, two recent metanalyses of stool DNA tests, one specifically examining methylation in stool DNA, have demonstrated that in pooled cohorts of 2,356 and 5,876 individuals, no marker was superior to current screening tools in unselected populations, although methylation markers performed better than mutation markers (sensitivity 75% versus 67%, specificity 91% versus 94%, respectively), all be it across different effects models (methylation fixed model; mutation random model)[336, 337].

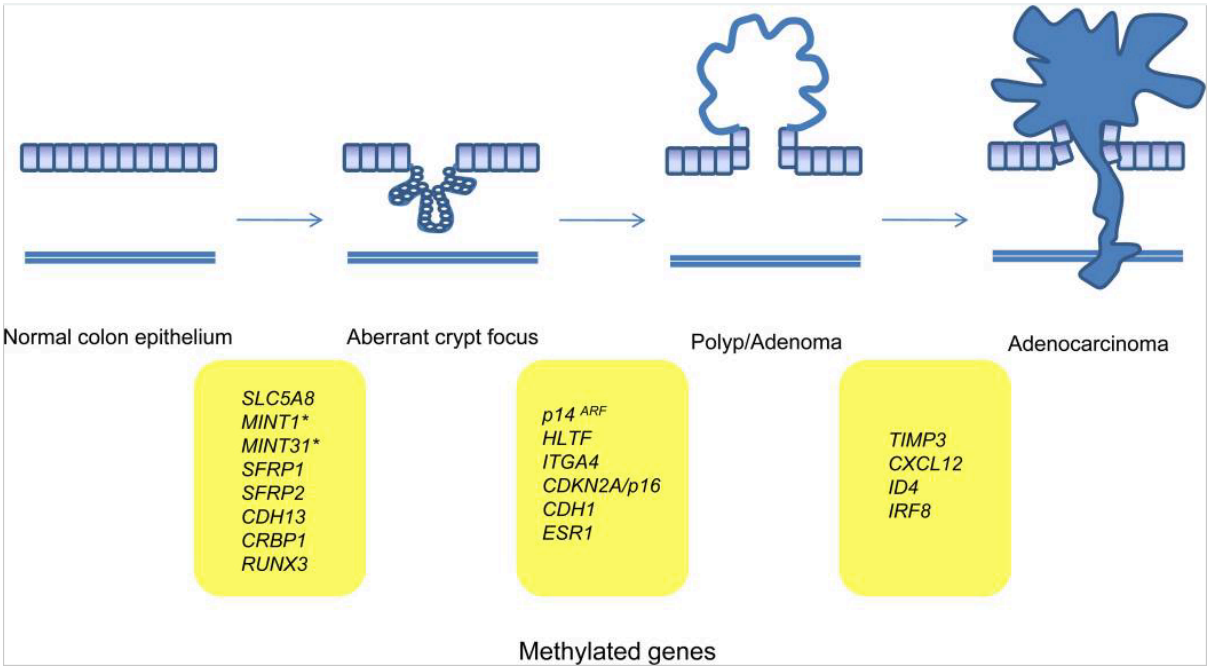
Currently, there are no methylation-based serum or stool DNA tests that are used in the screening or diagnosis of CRC, including mSEPT9, and most guidelines do not directly address DNA based testing of any sort[338]. Future evaluation of methylation-based markers such as hypermethylated GFRA1 and GSTM2, which were recently identified as potential diagnostic biomarkers in a genome-wide expressional analysis by Wei, may eventually prove fruitful if they can be translated into clinically utilisable tools[339].

3.1.3.3 Staging & Prognosis

As previously discussed, there are multiple genes and other loci where aberrations in methylation have been associated with the development of colorectal cancer, and a growing body of evidence suggesting specific hypermethylation events occurring on the classical adenoma-to-carcinoma pathway, as well as the development of tumour metastasis[307]. Examples of genes that are frequently found to be methylated in the early stages of dysplasia,

including in aberrant crypt foci, are ITGA4, MGMT, SCL5A8, FFRP2, and MINT1[340-343]. Interesting, the study by Kim and later confirmed by Oster, suggests that hypermethylation at specific genes is predominantly an early feature in the adenoma-to-carcinoma pathway, as many of the hypermethylation events that are present in cancers are already present in early and late adenomas (**Figure 3.4**)[343, 344]. LINE-1 hypomethylation has also been shown to be associated with early adenomatous changes in the colorectal mucosa, and increased with a linear relationship to disease progression (adenoma-carcinoma-metastasis)[345]. As well as individually hypermethylated genes or loci, the CIMP phenotype is also detectable in late adenomas, particularly of the serrated subtype, but is generally not present in earlier lesions, leading to the speculation that this is a gateway hypermethylation event in the development of more advanced disease[346]. Despite the identification of hypermethylated genes and application of the CIMP phenotype, no reliable method of discriminating between hyperplastic polyps and sessile serrated adenomas has been reliably developed, partially because of the heterogeneity in CIMP studies already discussed in section **1.3.6**. As dysplastic polyps transform to invasive cancers further hypermethylation occurs at conserved sites, specifically CXLC12, TIMP3, ID4, and IRF8, although these markers have not yet proved adequate for risk-stratifying and guiding the subsequent management of resected polpys[347].

Figure 3.4 Aberrant Hypermethylation Events in the Adenoma-to-Carcinoma Pathway (adapted from Lao & Grady)[307].



From a prognostic perspective, some aberrant methylation events have been associated with inferring an improved survival, such as MGMT and MLH1 hypermethylation, although hypermethylation of APC and *p14* may infer a survival disadvantage[348, 349]. Additionally, meta-analysis of 3440 CRC patients found that hypermethylation of p16 gene CDKN2A to be associated with a worse OS (HR 1.49, CI 1.28-1.74), as well as with lymphovascular invasion (HR 1.68, CI 1.15-2.47) and lymph node metastasis (HR 1.68, CI 1.09-2.59)[350]. Hypermethylation of HOPX-β, IGFBP3, EVL, CD109, and FLNC has also been associated with a worse prognosis, although the association to disease stage is less well defined in these cases[306, 351].

Since Toyota identified and first described CIMP, much of the focus relating to outcomes in CRC has been focused on methylator phenotypes rather than specific loci[177, 317]. CIMP is known to be associated with differing clinical and molecular characteristics in colorectal cancer and has previously been linked to outcomes when associated with MSI status and BRAF mutation[178, 352]. These studies have not, however, demonstrated an independent relationship between CIMP and outcomes for a number of reasons; firstly, due to lack of statistical power and the necessity of compound modelling, but more importantly due to the heterogeneous methodologies employed in determining CIMP. In Dahlin's study of 604 patients from two Swedish databases, CIMP-L was found to be associated with poor survival compared to CIMP-negative irrespective of microsatellite status, but inconsistently across cohorts in MSS groups. CIMP-H was also found to have a worse prognosis in the MSS groups, but the effect was lost in pooled analysis[352]. No relationship was evident between CIMP and cancer specific survival in the MSI pools. In this study the proportion of CIMP-negative patients was approximately 50%, with CIMP-L and CIMP-H contributing 35% and 15%, respectively. MSI was associated with CIMP-H, although statistical significance for RR was not achieved due to the small numbers involved (HR not interpretable on multivariate analysis). Although the datasets and the methodology of assessing methylation at each gene promotor region (bisulfite conversion and MethlyLight Q-RT-PCR) in this study are robust, the categorisation into CIMP status is essentially arbitrary; based on a simple and non-validated system of categorised cumulative numbers of methylated genes (CIMP-negative = 0 genes methylated; CIMP-L = 1-5 genes methylated; CIMP-H = 6-8 genes methylated).

To further investigate the relationship between CIMP and survival outcomes Juo performed a meta-analysis of 33 studies including 10,635 patients which demonstrated that CIMP is significantly associated with shorter DFS (pooled HR 1.45, CI 1.07-1.97) and OS (pooled HR 1.43, CI 1.18-1.73) irrespective of microsatellite instability[353]. The same study also examined survival following 5FU therapy based on CIMP status and found directly contradicting evidence for both survival advantage and disadvantage in different studies that lead to non-significance in meta-analysis. Although a strong indicator for CIMP being associated with poor outcomes in CRC, Juo's meta-analysis demonstrated further that there is a high degree of heterogeneity in the patient cohorts included in analysis, and in the methods by which CIMP is classified. Of the 33 studies included, 21 employed a dichotomised CIMP classification while the remainder employed a trichotomized methodology, and there was an even greater range of genes within each panel (ranging from 3 to 13 individual genes/loci), as well as methods for determining methylation status at each locus (described by Juo as, "often chosen arbitrarily"). A pooled analysis of the different methodologies used to determine CIMP was performed by Jia and Guo in an attempt to discern if one panel was more associated with outcomes than another, but unfortunately no significant relationship between any one panel and clinical outcomes was discovered, although a consensus was reached that CIMP-positive as determined by any panel was likely to confer a worse survival outcome[317]. The authors speculate that the lack of superiority in one panel over another is due to the large number of and high degree of overlap in the genes used in each panel, although CACN1G, IGF2, NEUROG1, RUNX3, hMLH1, p16, MINT1, MINT2, and MINT31 were most commonly used.

Few studies specifically address the survival of rectal cancer patients in relation to CIMP status as most present mixed cohorts of colon and rectal cancers. Samowitz examines a population of 864 cancers where 103 were found to be CIMP-positive based on a dichotomized panel of five genes (MHL1, MINT1, MINT2, MINT31, and CDKN2A) where ≥2 loci being hypermethylated indicating CIMP-positive[318]. The authors chose this panel and system based on their previous use of the method in previous publications but do not offer further justification beyond stating that there is currently no consensus in determining CIMP[175, 354]. This study reports that the 5-year survival for CIMP-negative and CIMP-positive is 72.0% and 63.2%, respectively (p=0.04) with an unadjusted hazard rate ratio (HRR) of 1.43 (CI 1.01 – 2.01), although the HRR fell to 1.32 and 95% CI crossed 1.0 (0.88 – 1.97) on multivariate analysis.

In another study by Jo, 150 patients with locally advanced rectal cancer enrolled in two phase III clinical trials (CAO/ARO/AIO-94 and -04) were analysed for dichotomized CIMP status based on a panel of five genes (RUNX3, SOCS1, NEUROG1, IGF2, and CAGNA2G)[149]. CIMP status was associated with a worse 3- and 5-year disease-free survival for patients identified as CIMP-positive versus -negative (56% vs 0%, and 85% vs 75%, respectively; HR 5.5 (CI 2.1 – 13.9). In this study, CIMP-positive was based on ≥3/5 loci being hypermethylated and accounted for only 10% of the study population.

3.1.3.4 Response to Therapies

There are conflicting reports of the impact of epigenetic events in the response of CRC to chemo- and radiotherapy, and the field is plagued by the same inconsistencies in methodology that limit the analysis of methylation in diagnosis and outcomes. Williamson reviews the development of CIMP as a marker for outcomes in rectal cancer, specifically in response to neoadjuvant therapy, summarising the findings that both hypermethylation and hypomethylation may have important prognostic roles in defining the response of lower gastrointestinal cancers to CRT. However, as study cohorts are frequently a mix of colon and rectal cancers there is inconsistency in CIMP-determining methodologies and in definitions of response to therapy (such as tumour regression grades - TRG) there is no consensus[148].

Specific examples of aberrant methylation affecting response to therapies have however been reported, including hypermethylated MGMT which is found in approximately 40% of CRC. In a phase II study of 68 patients with metastatic disease who have exhausted other therapies, methylated MGMT has been associated with a response to dacarbazine, where no response was detected in patients with the non-methylated gene[355, 356]. Furthermore, Sun reports that a greater reduction of methylated MGMT in circulating tumour DNA was observed in patients demonstrating a good response to CRT (assessed by TRG) compared with those that did not respond well, although the number of participants in this study was extremely limited[357]. Molinari identified that methylation of the TIMP3 tumour suppressor gene was found to be different between tumours categorised on the basis of their TRG (ANOVA, $p<0.05$), although it was not differentially methylated between tumour and healthy tissues prior to treatment. The case series was also restricted to 74 patients, limiting its predictive value, and the only markers found to be differentially methylated between cancerous and healthy tissue were ESR1, CDH13, RARB, IGSF4, and APC, although none of these genes was predictive of response to

therapy or changed in methylation status following therapy[358]. In a study of 155 stage II and III tumour samples, Kawakami showed that LINE-1 hypomethylation was associated with a better response to adjuvant oral fluoropyrimidine therapy and overall survival compared to patients with high LINE-1 methylation[359].

As well as potential predictors of favourable response, methylation has also been identified as a predictor of poor response. In a study by Ebert, hypermethylation of the TFAP2E gene was associated with poor response to CRT irrespective of regime, demonstrating a less that 10% tumour-regression rate compared with 82% of tumours with hypomethylated gene($p<0.001$)[360]. Williamson also showed that CIMP assessed on the two-panel approach advocated by Kaneda and Yagi was significantly associated with EMVI, which itself was found to be an independent predictor of poor disease-related outcomes, in line with the wider literature[185, 186, 204]. However, in the previously discussed study by Jo, no association was found between CIMP status and tumour regression based on histological examination following neoadjuvant CRT and resection[149].

3.1.3.5 Summary

Methylation is emerging as an important biomarker in colorectal cancer. Its limitations are partly in the complexity of epigenetic events that are detectable in the carcinogenic pathway and discriminating between what is significant and what may be incidental. Additionally, divergence in methodologies for assessing individual gene promotor hypermethylation and in classifying CIMP has led to a lack of robustness in the evidence supporting its role CRC. This problem is not limited to CRC but is also a factor in defining the role of epigenetics in other tumour types, including gastric, breast, and many others[361-363]. The problems are however more acute in rectal cancer as it is most commonly included in broader cohorts of colorectal cancer; limiting the insight into what is increasingly becoming regarded as a separate disease entity and one that certainly has different management pathways.

3.2 Aims & Objectives

Rectal cancer is a disease that has a unique natural history and poses specific challenges to patients, clinicians, and scientists. Disease-specific outcomes for rectal cancer are worse than those for colonic cancers, as demonstrated by worse disease-free and overall survival. Rectal cancer also presents operative challenges that are generally not applicable to colonic tumours, especially when considering operating within the bony pelvis and constructing a "low" anastomosis. From a patients' perspective, pelvic surgery is frequently morbid and associated with the risk of significant side-effects and even death. Locally-advanced and locally-recurrent rectal tumours also frequently require neoadjuvant chemoradiotherapy (nCRT) to improve resection rates and reduce local recurrence, the administration of which is largely based on radiological staging. The presence of extramural vascular invasion (EMVI) is an important but inconsistently assessed component of this evaluation, meaning a proportion of patients with EMVI who may benefit from nCRT do not receive it. However, not all patients that are eligible for neoadjuvant therapy will benefit from it, and some may be better treated by primary surgery. Currently there is no method of predicting which tumours will respond to neoadjuvant therapy.

The role of epigenetic aberrations in colorectal cancer is not well defined. Methylation analysis, including CpG Island Methylator Phenotype (CIMP) classification, has been suggested as important in defining the pathogenesis, diagnosis, prognosis, and response to therapy of colorectal cancer; although currently there is no defining evidence of a clinically utilisable methylation-based investigation that is significant in managing individual patients with rectal cancer.

The general aims of this chapter are to investigate the methylation status of rectal cancers based upon CIMP and determine if there is any association to clinicopathological variables that may impact outcomes.

3.3 Material & Methods

3.3.1 Patient Selection, Data Collection, & Storage

One hundred consecutive rectal cancer patients were extracted from the prospectively maintained database as described in section **2.1.1** & **2.1.2**, and in accordance with the ethics laid out in section **2.1.3**. As previously stated, patients were neoadjuvant therapy naïve, and exclusion criteria also included patients with hereditary or other identifiable predispositions to carcinogenesis, such as inflammatory bowel disease. Patients who had received locoregional radiotherapy or prior chemotherapy within 10 years for other malignant diseases (such as radiotherapy for prostate cancer or previous chemotherapy for breast cancer) were also excluded. All patients underwent index surgery between January 2010 and May 2013 at a single centre (ABMU, Morriston Hospital, Swansea, SA6 6NL, UK) by a team of surgeons participating in the Swansea Pelvic Oncology MDT.

Tumour height was defined as low rectal (within 5cm of the anus), mid rectal (5-10cm form the anus), or high (≥10cm from the anus) dependent on the lowest extent of the tumour on initial MRI. Tumours at the rectosigmoid junction where the bulk of the tumour was located in the sigmoid, negating a TME excision, were excluded. Gender was dichotomised. Tumour characteristics were defined as per the ACPGBI or Royal College of Pathology guidelines with respective relation to pre-operative (mTNM) or postoperative (pTNM) staging and other tumour characteristics (CRM, differentiation, EMVI)[35, 206]. All patients underwent full pre-operative staging as described by the ACPGBI guidelines; including computed tomography of thorax, abdomen, and pelvis; high-resolution magnetic resonance imaging of the pelvis; and in selected cases, endorectal ultrasound[206]. All cases were confirmed by biopsy taken pre-operatively at endoscopy and cases other than adenocarcinoma (squamous, melanoma, sarcoma, gastrointestinal stromal tumour) were excluded. Surgical treatment included anterior resection, abdomino-peroneal resection, and beyond-TME procedures in keeping with guidelines, patient choice, and expertise (see section **1.2**). Adjuvant chemotherapy was administered based on current guidelines and best-practice.

Investigators were blinded to individual and identifying patient data during sample processing and analysis by a process of anonymisation. After the core data clinical was collected and samples extracted from archive, information linking samples to patient information was randomised and only re-associated following completion of data collection to prevent bias. Patient identifiable data was stored on an encrypted and password protected spreadsheet, and

information linking specimens for laboratory analysis to individuals was kept in a locked filing cabinet away from the laboratory, in keeping with the principles of NIHR Good Clinical Practice (GCP) and the UK Policy Framework for Health and Social Care Research[364].

3.3.2 Tissue Collection

Tissue was extracted from the archives of ABMU Singleton Hospital Department of Pathology (Sketty Lane, Swansea, SA2 8QA, UK). Tissues were supplied in triplicate as fixed-in-formalin paraffin-embedded (FFPE) slides at 5µm thickness. Slides were stained with haematoxylin and eosin (H&E) and checked by consultant histopathologist to ensure that each contained a minimum of 60% tumour. Where tumour was less than 60% of slide tissue it was marked so it could be selectively dissected from the slide to provide a more representative sample of tumour for analysis. Three slides were used for each patient to ensure adequate tumour DNA extraction.

3.3.3 DNA Extraction

Slides underwent de-waxing by submersion in 100% xylene for 5 minutes at room temperature, repeated twice in the laboratory fume hood. Slides were then submerged in 100% ethanol for a further 5 minutes and allowed to air dry at room temperature. Tumour cells were then removed using a clean scalpel blade by scraping into a 1.5ml eppendorf microcentrifuge tube.

DNA from tissues was obtained using the MasterPure Complete DNA and RNA purification kit (Epicentre, Illumina, Wisconsin, USA). A mixture of 2 µl proteinase K and 300 µl of Tissue and Cell Lysis Solution was applied to each sample and vortexed thoroughly. This mixture was then incubated at 65°C overnight (minimum 12hrs) in a heater block and then re-vortexed the following day. This protocol is a slight deviation from the manufacturers protocol (longer incubation), as a prolonged period at 65°C was shown to increase DNA yield. Samples were then cooled to 37°C and 1 µl of RNase A was added to the sample and vortexed. This was incubated at 37°C for 60 minutes. The samples were placed on ice for 3-5 minutes before proceeding with DNA extraction.

DNA extraction was performed by adding 175 µl of MPC Protein Precipitation Reagent to 300 µl of lysed sample and vortexed vigorously for 10 seconds. The debris was pelleted by centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube, the pellet was discarded, and 500 µl of isopropanol

was added to the recovered supernatant. The eppendorf containing the supernatant was then inverted 40 times to mix contents and precipitate DNA.

The mixture was then centrifuged again 4°C for 10 minutes at ≥10,000 x g to pellet the DNA. The isopropanol was carefully poured off without dislodging the DNA pellet. The pellet was then rinsed gently twice with 70% ethanol. Residual ethanol was removed with a pipette and the samples were left to air dry for 10 minutes. The DNA was re-suspended in 35 µl of TE Buffer.

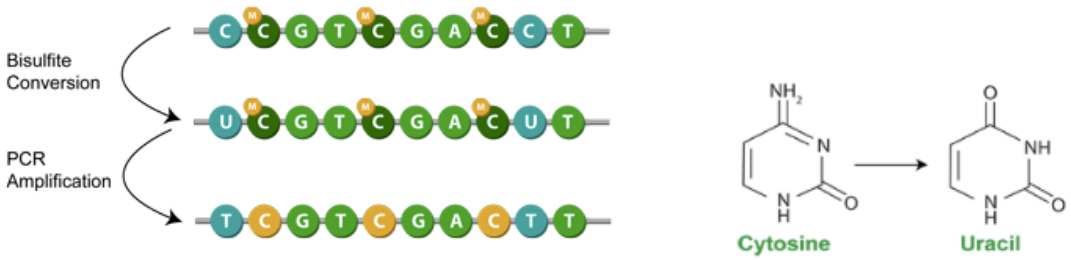
The quantity and quality of DNA was measured at absorbance between 230nm and 320nm using spectrophotometry (Nanodrop ND-1000, Software v 3.1.2, Thermoscientific, Delaware, USA). DNA quantity was calculated by multiplying the measured concentration following spectrophotometry at 260nm with the dilution factor. DNA was diluted to a working concentration of 20ng μl^{-1}. Purity was further assured by calculating the absorbance at 260nm to absorbance at 280nm ratio. Extracted DNA was stored at -20°C during active experimentation and archived at -80°C when not in use.

3.3.4 Bisulfite Conversion

Methylation specific PCR (msPCR) was carried out as a two-stage process of bisulfite-conversion and then amplification by bisulfite-specific primers, as described by Herman (**Figure 3.5**)[365]. The process of bisulfite-conversion first exchanges unmethylated cytosine in CpG islands for uracil, and then subjects the converted DNA to parallel PCR reactions with primers targeted at the CpG islands of the genes of interest; one primer amplifying CpG islands containing uracil, and the other amplifying native methylated cytosine. Genes with unmethylated CpG islands will amplify against the converted uracil primer, and those with methylated CpG islands will amplify against the cytosine primer.

Bisulfite conversion was performed using the Imprint DNA Modification Kit (Sigma Aldrich, St. Louis, USA). A two-step bisulfite-conversion protocol was employed as per the manufacturers recommendation as a means of producing high-quality DNA from a low-quantity input (100 pg to 10 ng); the first step performs bisulfite conversion and the second step is a post-modification clean-up to remove unconsumed reagents.

Figure 3.5 Bisulfite conversion & the nucleotides cytosine and uracil.



The DNA modification solution was first prepared by adding 1.1 ml of DNA Modification Solution to 1 vial of DNA Modification Powder. This mixture was vortexed for 2 minutes at room temperature and then 40 µL of Balance Solution was added, before a further brief vortex. The vial was examined for any undissolved particles was incubated at 65°C for 2 minutes if any were present, followed by further vortex. DNA was placed in a 1.5 ml micro-centrifuge tube and 110 µL of the prepared DNA modification solution was then added into the DNA and mixed by gentle pipetting. The mixture was the incubated at 99°C for 6 minutes and then at 65 °C for 90 minutes.

Clean-up was then performed by placing a spin column into a cap-less collection tube to which 300 µL of Capture Solution had been added and allowed to sit on the column for 1 minute. The modified DNA solution was then placed into the spin column and centrifuged at 12,000 x g for 20 seconds at 4°C. The flow-through was then discarded. 200 µL of an ethanol-diluted cleaning solution was the added to the spin column and centrifuged for 20 seconds. 50 µL of a balance/ethanol wash solution was added to the bottom of the spin column and incubated for 8 minutes at room temperature. After incubation, further centrifugation for 20 seconds was performed and the flow-through discarded. 200 µL of 90% ethanol solution was then added to the spin column and centrifuged for 20 seconds and the flow-through again discarded. The cap-less collection tube was then discarded, and the spin column placed into a 1.5 ml collection tube. 20 µL of elution solution was then added to the bottom of the spin column to extract the DNA from the column and return to solution. The spin column and elution solution were incubated for 1 minute at room temperature and then centrifuged for 20 seconds. The spin column was then discarded, and the eluted solution collected contained the purified bisulfite modified DNA. Modified DNA the proceeded directly to msPCR or was stored at –20°C for up to 2 months.

3.3.5 Methylation Specific PCR (msPCR)

All msPCR took place in the T100™ Thermal Cycler (Bio-Rad, Berkeley, CA, USA). Oligos and PCR conditions had previously been sourced and optimised by colleagues in-house during preceding research and from previously published studies and had also been further interrogated against methBlast bioinformatic software for target specificity[204, 365]. The genes targeted whose CpG islands are targeted are hMLH1, SOCS1, MINT1, THBD, IGFBP3, HAND1, ADAMTS1 and NEUROG1. Target genes were selected based on the Kaneda & Yagi system for CIMP assessment (see section **1.3.6** and **3.3.7**)[185]. For oligonucleotides refer to section **2.2.5**. Desalted oligonucleotide PCR primers (Sigma Aldrich, UK) were diluted to a working concentration of 10uM and stored at -20°C with all other PCR reagents. Conditions for each primer are set out in **Appendix I**. Preparation for each msPCR took place in laminar flow hoods cleaned with 70% ethanol solution and allowed to air dry under UV light to reduce the risk of DNA cross contamination or fouling of reactions. PCR-grade filtered pipette tips were used for handling DNA and reagents, and reactions were performed in micro-eppendorfs. msPCR reactions were carried out in a volume of 25 µL per sample as per Table 3.1.

Table 3.1 msPCR reagents.

Constituent	**Volume**
GoTaq® Hot Start Green Master Mix (Promega, Madison, WI, USA)	12.5 µL
Nuclease Free Water (Ambion)	10.5 µL
Forward Primer (200 nM)	0.5 µL
Reverse Primer (200nM)	0.5 µL
Bisulfite Converted DNA (40ng/µL)	2 µL

msPCR utilised a hot-start protocol consisting of an 8-minute hot start at 95°C followed by denaturing at 95°C for 30 seconds, then annealing at temperatures and durations specifically optimised to the primers (typically 30 to 37 seconds and 50-61 ^{O}C). An extension phase at 72°C followed and the cycle repeated. Positive controls for both the methylated and unmethylated DNA (Promega, UK) and a negative control were used throughout.

3.3.6 Visualisation of PCR Products

msPCR products were visualised by gel electrophoresis on 6% polyacrylamide gels against a 100bp DNA ladder containing 11 dsDNA fragments of 100-1000bp (in 100bp steps) & 1500bp (Promega, Madison, WI, USA). Hand formed 15-lane 1.5mm polyacrylamide gels were made in duplicate according to the following recipe: 16 ml dH_2O, 2.25 ml 10x TBE, 4 ml acrylamide, 110 µl 10% APS, 22 µl TEMED. This mixture was mixed by pipetting and transferred to two gel casts and allowed to polymerise for 60 minutes. Gels were then submerged vertically in electrophoresis tanks containing 1x TBE buffer and 10µL PCR product was mixed with 2 µL coloured loading buffer (RETROscript, Invitrogen) and then loaded into each well. Electrophoresis was performed at 170V, 400mA for 25 minutes or until the loading dye had reached the bottom of the plate.

Following electrophoresis, the gels were separated from their glass casting plates and stained by sequentially submerging into silver nitrate solution (1g/L) for 7 minutes and then submerging in formaldehyde & sodium hydroxide solution for 2 minutes until visualisation of bands was achieved. Gels are then rinsed with dH_2O and photographed in white light using Gel Doc XR (Bio-Rad) system and Quantity One software to adjust the brightness and size of the image (Bio-Rad). Gels were then discarded into a hazardous waste container.

3.3.7 CIMP Classification

CIMP status was classified according to the methodology described by Kaneda & Yagi[185]. This method was chosen due to the approach taken to classifying CIMP status, as discussed in section **1.3.6**. The epigenotyping system is again described below.

- CIMP-High if ≥ 2/3 of Group 1 markers are methylated, irrespective of Group 2 markers
- CIMP-Intermediate if 1/3 Group 1 markers and ≥3/5 Group 2 markers is methylated
- CIMP-Low if ≤1 Group 1 markers is methylated and ≤2 Group 2 markers is methylated

3.3.8 Statistical Analysis

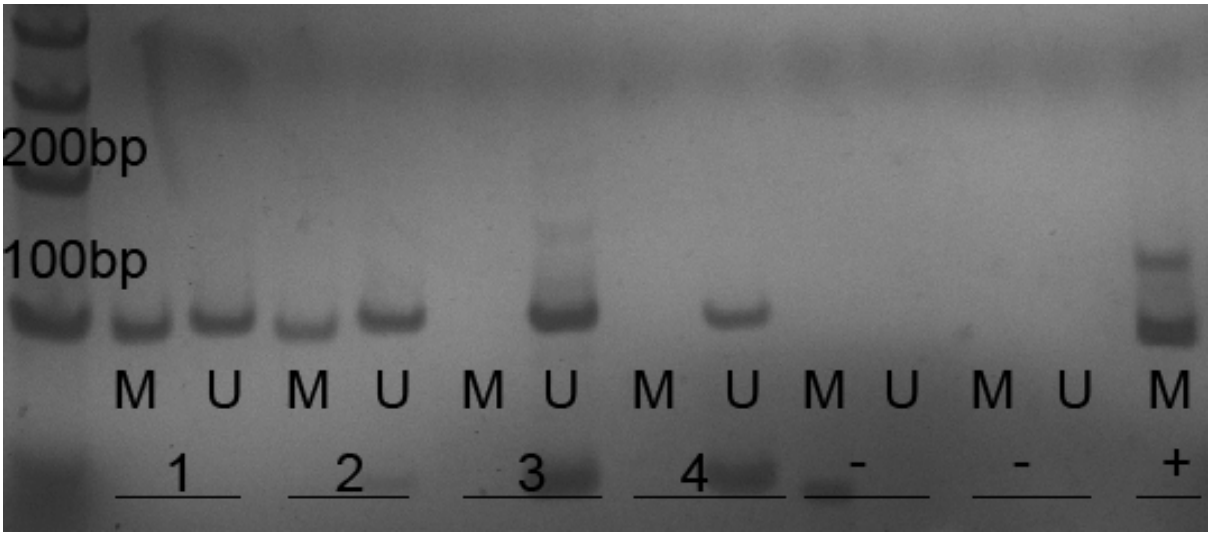
Data analysis was performed using the Microsoft Office Excel (v.16.12) and the XLStat Addinsoft (v.2017) plug-in (macro). Results were expressed as mean +/- standard error of the mean (SEM) where appropriate. Normalcy of data distribution was determined using a Kolmogorov-Smirnov test. If data were normally distributed, statistical significance was determined using Student's t-test for single comparisons or one-way ANOVA where more than one comparison was made. If data were not normally distributed, Mann-Whitney's test was used to compare unpaired means and Wilcoxon test used for paired data. Log-Rank (Mantel-Cox) tests were used where data was right-side skewed and censored. Comparison of expected frequencies was performed with two-tailed Chi Squared or Fishers exact test if observed events were less than 10. Survival curves were calculated using Kaplan-Meier estimate. The null hypothesis was rejected at the level of 5% ($p>0.05$).

3.4 Results

3.4.1 Patient and Tumour Characteristics

There were 100 patients included in this study (44 patients with an EMVI-positive tumour and 56 with no evidence of EMVI). Seventy patients were male, with a statistically significant proportion of male patients demonstrating EMVI positivity (82% positive vs 61%, p=0.022). The median age was 70 years (range 45 – 89 years), and patients were of a similar age in both groups. Nineteen patients had died at the time analysis was undertaken (01/09/2017). Median follow up was 54.2 months (IQR 25 – 68 months). Mean overall DFS and OS were 29 and 34 months, respectively. When differences between both groups were analysed (**Table 3.2**), patients with EMVI-positive tumours had more advanced pathological staging by pT, pN, and AJCC classifications (p=0.002, p=<0.0001, and p-<0.0001, respectively). There was a corresponding association between EMVI-positive tumours and the need for adjuvant chemotherapy (p=<0.0001). There was, however, no statistically significant difference in CRM positivity (p=0.86), tumour perforation (p=0.07) or tumour differentiation (p=0.52).

Figure 3.6 Resolved polyacrylamide gel demonstrating tandem-PRC products for methylated (M) & unmethylated (U) primers against samples (1-4) with controls & DNA ladder. Samples 1 & 2 were methylated (strong M bands as well as U bands), samples 3 & 4 unmethylated (U bands only). Two negative control reactions (no template DNA) and a positive control lane (hypermethylated DNA) are also shown.



3.4.2 CIMP Classification & EMVI

There were a total of 51 patients with CIMP-L, 48 patients with CIMP-I, and one patient with CIMP-H phenotype (**Table 3.3**). The assessment of 40 patients' CIMP status was conducted by a co-investigator, although all subsequent analysis was performed by the author. Chi-square (Pearson) demonstrated a positive correlation between EMVI-positive tumours and CIMP-intermediate epigenotype ($p=<0.001$). This effect was preserved if the single CIMP-high sample was amalgamated with the CIMP-intermediate group ($p=0.00014$) and did not significantly affect any of the regression analysis performed henceforth.

CIMP-intermediate labelling was associated with worse tumour pAJCC stage ($p=0.03$) and showed a trend towards association with the need for adjuvant chemotherapy ($p=0.055$), but was not associated with T or N stage, tumour height, sex, or CRM involvement (**Table 3.4**).

Table 3.2. Patient and Tumour Characteristics

	EMVI +ve (n=44)	**EMVI-ve (n=56)**	**P-value[1]**
Median Age (range)	72 (45-85)	69 (46-89)	0.86[2]
Sex (male)	36 (82%)	34(61%)	0.022*
Tumour Height			0.34
Upper (10-15cm)	8 (30%)	17 (27%)	
Mid (5-10cm)	23 (43%)	23 (47%)	
Lower (0-5cm)	13 (27%)	16 (27%)	
pT			0.002*
T1	0 (0%)	1 (2%)	
T2	2 (5%)	12 (21%)	
T3	31 (75%)	41 (70%)	
T4	11 (20%)	2 (7%)	
pN			<0.0001*
N0	8 (20%)	42 (73%)	
N1	21 (46%)	7 (14%)	
N2	15 (34%)	7 (13%)	
pAJCC			<0.0001*
1	0 (0%)	8 (27%)	
2	8 (16%)	37 (41%)	
3	32 (75%)	9 (27%)	
4	4 (9%)	2 (5%)	
CRM +ve	1 (3%)	1 (3%)	0.86
Tumour Perforation	6 (7%)	2 (9%)	0.07
Tumour Differentiation			0.52
Poor	6 (14%)	5 (9%)	
Moderate	38 (87%)	50 (89%)	
Well	0 (0%)	1 (2%)	
Non-Restorative Operation	18 (27%)	20 (46%)	0.60
Adjuvant Chemotherapy	31 (68%)	10 (16%)	<0.0001*
Systemic Recurrence	15 (30%)	11 (13%)	0.10
Local Recurrence	3 (7%)	3 (5%)	0.76

[1]Pearson's Chi Square. EMVI, Extramural Vascular Invasion; pAJCC, American Joint Cancer Committee pathological stage; CRM, circumferential resection margin; OS, Overall Survival; DFS; Disease Free Survival.

Table 3.3 CIMP Classification and EMVI status

Contingency Distribution	**EMVI +ve (n=44)**	**EMVI-ve (n=56)**	**Distribution p= <0.001[1]**
CIMP-low	13 (30%)	38 (68%)	
CIMP-intermediate	31 (70%)	17 (30%)	
CIMP-high	0 (0%)	1 (2%)	

[1]Pearson Chi-Square. CIMP, CpG Island Methylator Epigenotype; EMVI, Extramural Vascular Invasion.

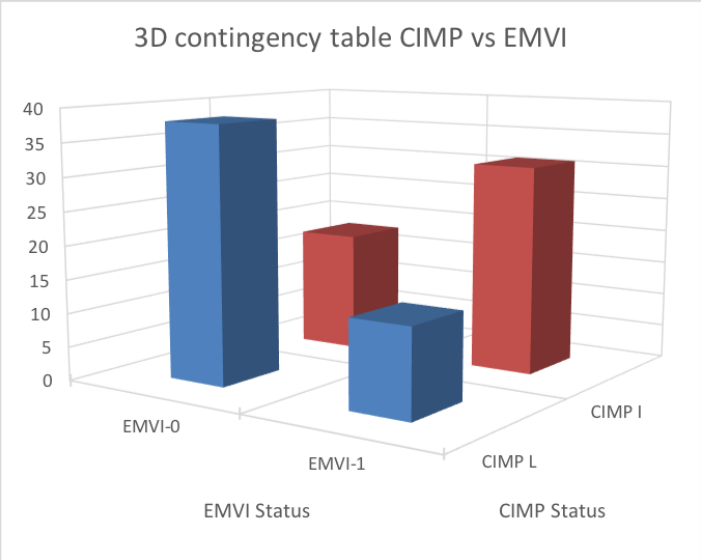


Table 3.4. CIMP status and association with patient and tumour factors.

	CIMP-I (n=48)	**CIMP-L (n=51)**	**P-value**
Median Age (range)	67 (45-89)	70 (47-89)	0.86[2]
Sex (male)	33 (68.8%)	36 (70.6%)	0.42
Tumour Height			0.33
Upper (10-15cm)	13 (27.1%)	12 (23.5%)	
Mid (5-10cm)	25 (52.1%)	21 (41.2%)	
Lower (0-5cm)	10 (20.8%)	18 (35.3%)	
pT			0.17
T1	0 (0.0%)	1 (2.0%)	
T2	4 (8.3%)	9 (17.6%)	
T3	36 (75.0%)	36 (70.6%)	
T4	8 (16.7%)	5 (9.8%)	
pN			0.28
N0	24 (50%)	26 (51.0%)	
N1	17 (35.4%)	11 (21.6%)	
N2	7 (14.6%)	14 (27.5%)	
pAJCC			0.03*
1	0 (0.0%)	8 (15.7%)	
2	24 (50.0%)	21 (41.2%)	
3	20 (41.7%)	20 (39.2%)	
4	4 (8.3%)	2 (3.9%)	
CRM +ve	2 (4.2%)	0 (0.0%)	0.15
Tumour Perforation	4 (8.3%)	4 (7.8%)	0.98
Tumour Differentiation			0.58
Poor	5 (10.4%)	6 (11.8%)	
Moderate	43 (89.6%)	44 (86.3%)	
Well	0 (0.0%)	1 (2.0%)	
Non-Restorative Operation	16 (33.3%)	22 (43.1%)	0.25
Adjuvant Chemotherapy	25 (52.1%)	16 (31.4%)	0.055
Systemic Recurrence	13 (27.1%)	7 (13.7%)	0.61
Local Recurrence	3 (6.3%)	3 (5.9%)	0.98

[1]Pearson's Chi Square, [2]Students T-Test. EMVI, Extramural Vascular Invasion; pAJCC, American Joint Cancer Committee pathological stage; CRM, circumferential resection margin; OS, Overall Survival; DFS; Disease Free Survival.

Using a multivariable logistic regression model (**Table 3.5**), CIMP status remained a highly significant predictor of EMVI status (p=0.001), independent of pAJCC staging (p=<0.001). When considering the development of systemic recurrence, only the presence of a perforated tumour was significantly associated (p=0.038). Only EMVI positivity was associated with poor DFS (p=0.038), but not significantly associated with poor OS, although trend was observed (p=0.08) for both EMVI positivity and CIMP-I status (p=0.08) (**Figure 3.6**).

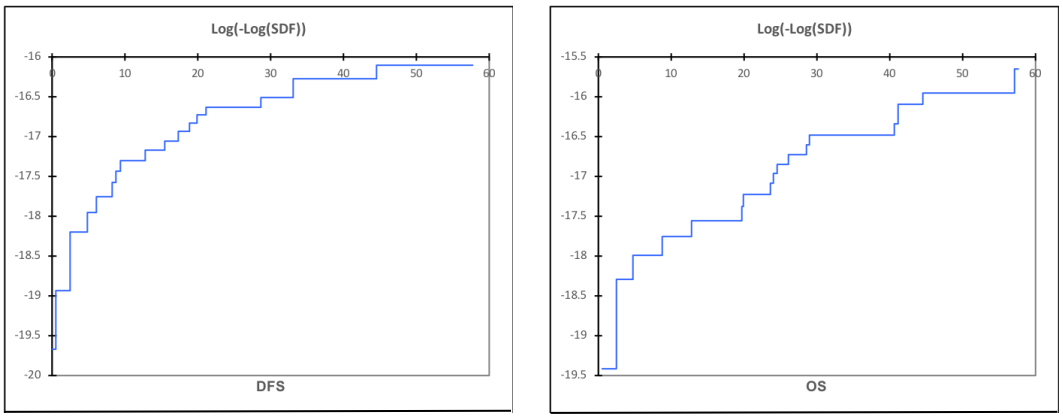
Table 3.5 Multivariable Logistic Regression analysis of factors associated with EMVI, Metastatic Disease, and Overall & Disease-Free Survival: Standard Coefficients (SC) & Hazard Ratios (HR).

	Factor	**SC or HR (95% CI)**	**P value**
EMVI	CIMP Intermediate	1.27 (0.55-1.91)	0.001*[1]
	Male Sex	-0.13 (-0.59-0.34)	0.59[1]
	Increasing pAJCC stage	1.17 (0.59-1.76)	<0.001*[1]
	CRM positivity	0.09 (-0.32-0.50)	0.66[1]
	Perforated Tumour	0.21 (-0.26-0.68)	0.38[1]
	Non-restorative Operation	-0.27 (-0.75-0.20)	0.26[1]
Systemic Recurrence	CIMP Intermediate	0.78 (-0.20-1.76)	0.12[1]
	Male Sex	-0.79 (-1.81-0.24)	0.13[1]
	Increasing pAJCC stage	-0.36 (-1.46-0.73)	0.51[1]
	CRM positivity	0.14 (-0.29-0.57)	0.53[1]
	Perforated Tumour	0.54 (0.04-1.04)	0.034*[1]
	Non-restorative Operation	0.56 (-0.19-1.31)	0.14[1]
Overall Survival (OS)	CIMP Intermediate	0.36 (0.12-1.12)	0.08[2]
	EMVI Positive	4.59 (0.85-24.69)	0.08[2]
	Increasing pAJCC stage		0.91[2]
	Perforated Tumour		0.99[2]
Disease-Free Survival (DFS)	CIMP Intermediate	0.39 (0.13-1.18)	0.10[2]
	EMVI Positive	5.98 (1.10-32.50)	0.038*[2]
	Increasing pAJCC stage		1.0[2]
	Perforated Tumour		1.0[2]

[1]Binary Logistic Regression, [2]Cox Proportional Hazard. CIMP, CpG Island Methylator Phenotype, EMVI, Extramural Vascular Invasion; pAJCC, American Joint Cancer Committee pathological stage; CRM, circumferential resection margin; OS, Overall Survival; DFS; Disease Free Survival.

Figure 3.7 Cox log(-log(survival)) analysis for EMVI versus DFS (a) and OS (b), demonstrating a significant association with poor DFS (left-shift, p=0.038), but not OS (insignificant but trend towards left-shift, p=0.08).

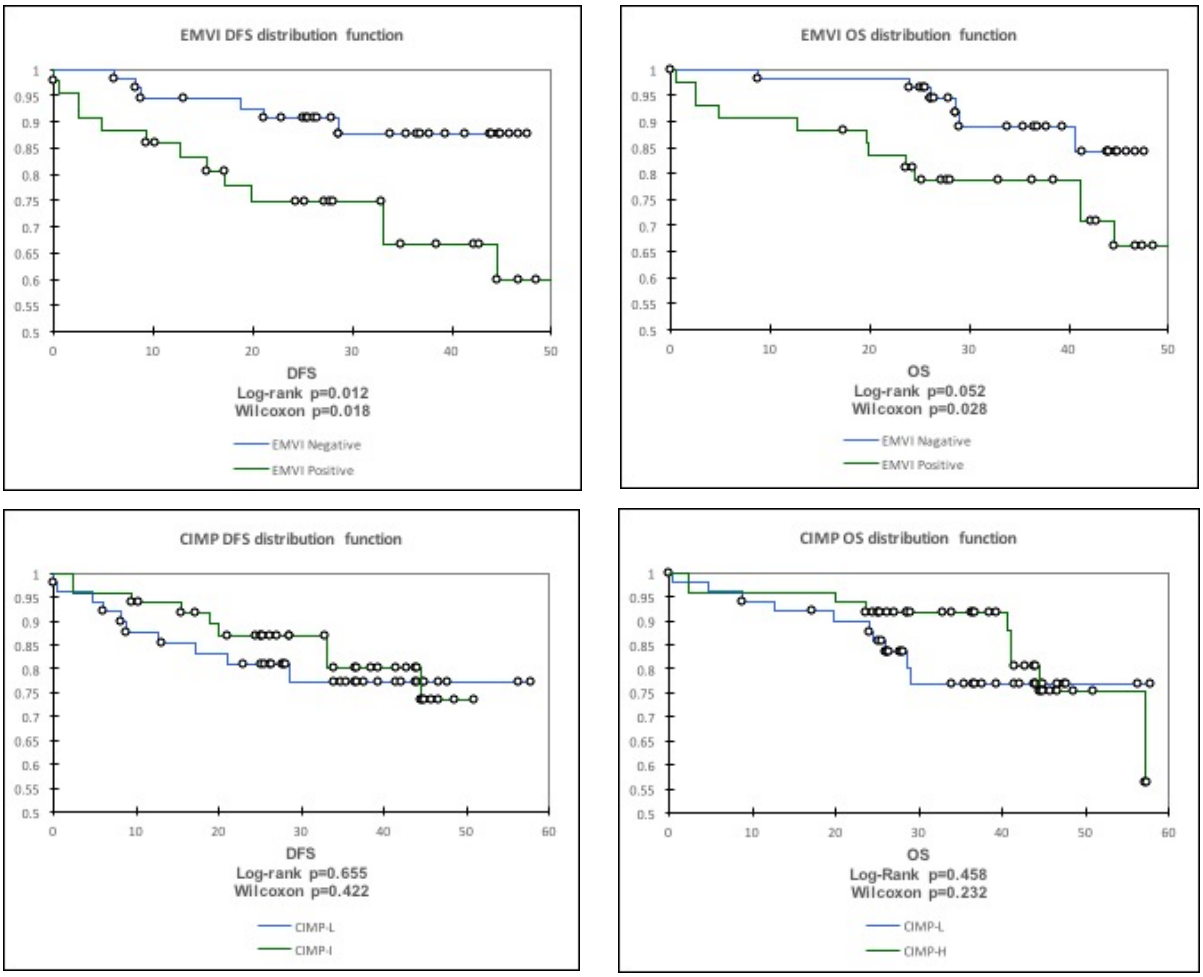
a. b.



3.4.3 Survival Analysis

Kaplan-Meier analysis (**Figure 3.7**) demonstrated that overall survival (OS) and disease-free survival (DFS) were greater in the EMVI-negative group, but only reached statistical significance for DFS, although strong trend was observed for OS (p=0.012 and p=0.52 respectively, by log-rank). This effect was however overcome by Gehan-Breslow-Wilcoxon method (which weighs positively for early deaths (p=0.018 and p=0.028 for DFS and OS, respectively). CIMP status was not statistically related to DFS or OS, although CIMP and OS approached significance at p=0.08. There was a total of 20 patients with systemic metastases (two of these had synchronous metastases) and six had local recurrence. Although a significantly greater number of patients with EMVI-positive tumours received adjuvant chemotherapy (p<0.0001), this relationship was not observed for CIMP-intermediate tumours, although trend was observed (p=0.055). Significance was not achieved through a re-sampling (bootstrapping) technique.

Figure 3.8 Kaplan-Meier overall and disease-free survival analysis for EMVI and CIMP.



3.5 Discussion

The aim of this chapter was to investigate the methylation status of rectal cancers based upon CIMP and determine if there was any association with EMVI or other clinicopathological variables that may impact outcomes. The findings are considered in the context of the poorer outcomes from rectal cancer in terms of local and systemic recurrence, and survival.

A clear association was demonstrated between the presence of pEMVI and male sex, T and N stage, AJCC classification, and the requirement for adjuvant chemotherapy. EMVI has previously been shown to be an independent risk factor for systemic and local recurrence, and death; and is an indication for adjuvant chemotherapy[276-278, 293]. This study confirms the association of EMVI with worse clinicopathological features, and on Kaplan-Meier analysis, these translated into statistically significant survival disadvantage, in keeping with the findings of Smith (**Figure 3.3**)[280]. Currently, neoadjuvant therapy from rectal cancers is advised on the basis of threatened circumferential resection margins as demonstrated on preoperative radiological imaging, but tumour genetic and epigenetic analysis is not routinely part of pre-therapeutic assessment[85]. By associating CIMP epigenotype with EMVI, the possibility of utilising neoadjuvant therapies in patients who are not demonstrated to have locally advanced disease by radiological standards, but who may be judged to be at higher risk based on epigenetic tumour profiling, may improve outcomes. For this reason, it is important to understand the role that epigenetic phenotypes may have on tumour behaviour so that they may be harnessed to therapeutic effect and inform clinical decision making.

Although a significant relationship was demonstrated between CIMP-intermediate status and EMVI positivity, this was not translated in to a disease-free or overall survival disadvantage for CIMP-intermediate patients based on Kaplan-Meier analysis, despite EMVI-positivity being an independent risk-factor for disease recurrence (Cox Proportional Hazard r=5.98 (1.10-32.50), p=0.038). These findings are in keeping with prior results in a similar cohort from our own unit, which indicate a positive correlation between CIMP-high status and EMVI in a series of 160 rectal cancers undergoing neoadjuvant treatment; although this did not translate into a significant relationship to survival[204]. There was also no significant relationship between KRAS or BRAF and CIMP. More broadly, results published by Kim also demonstrated that a higher CIMP status was associated with worse DFS, but only for colonic tumours as opposed to rectal tumours based on a mixed cohort of 157 patients[366]. In another study, high CIMP epigenotype was also found to be associated with worse overall- and progression-free survival

in patients with metastatic colorectal cancer, although this study also included both colonic and rectal tumours[367]. Each of these studies are contributory to the hypothesis that CIMP positivity is associated with more locally advanced tumour types and poorer clinical outcomes, although the relationship is not straightforward. In a review of 20 heterogeneous studies, Gallois discusses the prognostic value of EMVI in mixed cohorts of colorectal cancers, concluding that currently there is not sufficient evidence to support EMVI as a prognostic indicator[368]. Despite the differences between the panels used and mixed cohorts, what is clear is that the relationship between CIMP status and clinical outcomes is not solely limited to the relationship to EMVI, else DFS and OS outcomes would be more closely aligned; pointing towards additional roles for methylation in processes such as response to CRT, cell death, and tumour-immunological interaction.

The consensus molecular subtyping (CMS) of colorectal cancers has identified 4 distinct tumour subtypes based on molecular analysis, with CMS1 being defined as typically hypermethylated and associated with the serrated pathway in the proximal large bowel, rather than in the rectum[118, 201]. In the original CMS study, the frequency of CMS1 reported in the rectum was 3%. Considering this, the dataset in this study of 100 consecutive patients with rectal cancers, given exclusions, demonstrated a higher than expected proportion of CIMP-intermediate and -high (49%) which may be considered as analogous to CMS1 tumours. Although the CIMP-I and CIMP-H groups were considered as one for the purposes of analysis (equivalent to CIMP-positive in other studies), this finding does question the assumption that hypermethylation is a predominantly right colonic finding associated with the serrated pathway and may therefore represent an under-reported subgroup of rectal cancers. These findings must be considered in the context of the methodology chosen to determine CIMP (Kaneda & Yagi), although as previously discussed, there is no consensus on determining CIMP and the methodology employed in this study was chosen for its robustness and use throughout other contemporaneous investigations, although other methods may have returned a lower frequency[185, 186].

One reason for the discrepancy is that the cohorts examined are not alike in several ways, especially when considering the relatively selective cohort used in this chapter (exclusion of patients who had received neoadjuvant therapy, for example) compared to the inclusive nature of the Guinney study which was based on eighteen public and proprietary colorectal cancer datasets comprising 4,151 patients[201]. However, although the Guinney dataset is much larger,

only 966 patients (23%) had extractable methylation data available for contribution to the subtyping algorithms, raising the possibility of this component being under-represented when compared to other molecular markers in the modelling. Furthermore, the assessment of CIMP employed by Guinney was also atypical; relying on comparative sub-group methylation analysis to determine which loci would be best at discriminating between the putative molecular subtypes, and then augmenting the effect within the model to facilitate discrimination, as opposed to a standardised and blinded assessment of methylation status at previously identified CpG islands and thus determine CIMP status.

Although patients receiving neoadjuvant therapy were excluded in our study, the relationship between methylation and response to CRT (neoadjuvant and adjuvant) is likely to be significant, as has been identified in previous studies[204, 369]. The benefits of neoadjuvant therapy in rectal cancer are clear, but it is acknowledged that a tumour's response to neoadjuvant therapy is currently not predictable, and identifying significant factors that affect response may be beneficial in managing patients[370]. Currently, mucinous tumours and poorly differentiated tumours have all been associated with poor response to neoadjuvant therapy, but the prognostic values of these measures is limited and has no clinical utility in restricting access to pre-surgical therapies[371]. Unfortunately this means that a proportion of patients who undergo neoadjuvant therapy will gain no benefit, and may potentially come to harm as a result of systemic chemotherapy and/or local radiotherapy, and/or miss the opportunity to have a surgical intervention[372]. For these reasons there is an urgency in identifying reliable molecular markers of tumour response, including exploration of the methylome for significant relationships[373-375]. One successful example of this approach is the utilisation of a single nucleotide mutation in the KRAS gene in predicting the response of patients to adjuvant therapy using epidermal growth factor receptor (EGFR) inhibitors; which are increasingly utilised in order to increase the effectiveness of treatment[376]. With regards methylation, Yokoi reported that DNA methylation may play an important role in affecting response to radiotherapy in an *in vitro* colorectal cell line model[377]. This process was dependent on methylation-controlled expression of cellular retinol binding protein 1; cellular response to radiotherapy being strongly related to expression. In our study, CIMP-intermediate was associated with a trend towards requirement for adjuvant chemotherapy (p=0.055), although it did not reach significance and was not an independent risk factor for DFS or OS. Studies examining the correlation between CIMP status and response to CRT have shown some promise, although the results have been inconsistent and are plagued by methodological

inconsistencies[378-380]. Further studies have identified methylation to be important in the response of tumours to chemoradiotherapy in other organ systems, including lung, breast, glioma, and others; and is a field that warrants further investigation in the context of rectal cancers[381-386]. The response of tumours to neoadjuvant therapy is especially important when considering the challenges of predicting EMVI on pre-operative imaging. MRI has been shown to be accurate in the local staging rectal tumours and indicating where circumferential resection margins are threatened, thus indicating the need for neoadjuvant therapy, but there is an acknowledged shortcoming in the sensitivity and specificity of MRI-detected EMVI, especially where EMVI is present in vessels smaller than 3mm or when EMVI volume is low [281, 282, 387,]. If the prognostic validity of CIMP status could be established based on biopsy, it may open the opportunity to intervene on radiologically EMVI-negative but CIMP-intermediate/-high and pathologically unfavourable tumours before surgery.

3.6 Conclusion

A clear relationship was demonstrated between CIMP-positive (pooled CIMP-intermediate and -high) and EMVI. The CIMP-positive pool may be considered as analogous to CMS1, although the number of cases reported in our series was significantly higher than that expected given the frequency of CMS1 in the previously published data. EMVI-positive tumours demonstrated worse DFS and OS, and were associated with poorer clinicopathological features, although this disadvantage was not conferred to CIMP-positive tumours, suggesting a more complex relationship between EMVI, CIMP, and outcomes. A better understanding of the biological mechanisms underlying CIMP and EMVI may help illuminate the clinicopathological relationship between EMVI and CIMP and prove useful in stratifying and guiding disease management. This may be especially important for determining patients who may be at risk of developing early local or distant disease recurrence and therefore benefit from systemic therapy, either before or after surgery, and who may not have obvious evidence of EMVI on initial staging.

Chapter 4

The Biological Effects of Demethylation on Colorectal Cancer Cells *In Vitro*.

4.1 Introduction

Methylation is emerging as an important factor in carcinogenesis of colorectal cancer, as well as other cancers[309, 388, 389]. Human tissue studies have identified specific epigenetic changes in colorectal cancers, many of which have putative cellular effects that may be directly contributory to the carcinogenic process; such as the hypermethylation events identified in the adenoma-to-carcinoma model and the *Wnt* pathway[307, 309]. Other methylation markers identified in colorectal cancer are less obviously linked to carcinogenesis but may act as proxies for assessing the overall methylation status of DNA in healthy and diseased states. Assessment of methylation is important in understanding colorectal cancer pathogenesis and in classifying colorectal cancers; demonstrated by methylation being an integral part of the classification systems employed to characterise colorectal cancer[118, 161, 185]. As DNA methylation is an active and dynamic cellular process, important in the normal homeostasis of cells and phenotypic differentiation, it is open to modification *in vitro* and *in vivo* for experimental and, potentially, therapeutic purposes.

4.1.1 *In Vitro* Studies of Colorectal Cancer

The systematic investigation of colorectal cancer in the laboratory can be dated to at least 1940, when mice were fed diets containing potential carcinogens and the effects on the gastrointestinal mucosa examined at vivisection[390]. This work was succeeded by further studies in animals until experimental techniques in *in vitro* cell culture began to be developed in the 1960's, based first on cells extracted from viable tumours, and then on immortalised cell lines[391, 392]. Many of the themes of the current investigation of colorectal carcinogenesis were being explored in relatively early work during the 1970s, including the colonic microbiome, the role of dietary fibre and fats, and even the niche field of DNA alkylating agents derived from rocket propellants[393, 394]! It wasn't until the 1980s, however, that the culture of colorectal cell lines (healthy, adenomatous, & cancerous) became a viable and widespread technique,

partially due to the relative ease with which colorectal cancer cell lines may be propagated, compared to other cancer types[395-397]. The widespread adoption of cancer cell culture has greatly facilitated the mechanistic understanding of carcinogenesis, as well as drug discovery. For instance, the NCI-60 cancer cell line panel is a cohort of cell lines employed by the National Cancer Institute (USA) for high-volume screening of potential anti-cancer drugs against well established and characterised cell lines, including multiple colon cancers (original colon set: COLO 205, HCC-2998, HCT116, HCT-15, HT-29, KM12, SW-620; latter additions: DLD-1, KM20L2), and has aided in drug discovery (and arguably in early abandonment of futile investigation)[398].

Despite advances, there are however many shortcomings in *in vitro* modelling of colorectal cancer that limit its utility and possibly account for some of the failings in translating bench-top science into the clinic. New strategies are required to overcome these dilemmas. Specifically, regarding the investigation of rectal cancer as an individual disease entity separate from colorectal cancer, there is currently no commercially available rectal cancer-specific immortalised cell line available to investigators. This shortcoming, however, must be balanced against the consideration that within the cohort of rectal cancers are found many epigenotypes, genotypes, and phenotypes; as demonstrated by the consensus molecular subtyping systems, limiting the utility of a single rectum-derived cell line. Additionally, single-cell culture models of this manner do not account for the intratumoral heterogeneity displayed in rectal and other cancers[399]. Monoculture and 2D-culture are also limited in their modelling of *in vivo* cancers in that they fail to provide the complex inter-cell interactions and tumour microenvironmental factors at play in *in situ* tumours. This is particularly important when considering the important emerging role of immune responses to colorectal cancer, and the hypoxic conditions involved in cellular redox reactions[400]. Latterly, these challenges have been met with developments in tissue organoid culture (3D culture) and co-culture techniques in tissue engineering[401, 402]. Spheroid cell culture has been proposed as one model for better modelling tumour microenvironments and cellular interactions in colorectal cancer and has been performed with success in a number of cell lines. However, as a relatively novel method of tissue culture, new challenges such as divergent differentiation dependent on location within the spheroid and changes in cell behaviour when compared to 2D culture (e.g. propensity to invade) have emerged[403]. Despite its limitations and technical challenges, cell culture, whether 2D or 3D, is still well placed to facilitate the understanding of colorectal carcinogenesis and aid in

developing therapeutic strategies, especially when coupled to modern technologies in translational research[404].

4.1.2 Migration & Invasion Assays

4.1.2.1 Introduction

Cellular invasion and migration are two inter-related processes that are crucial in the pathogenesis of colorectal cancer, and are similarly rooted in the cellular events that facilitate epithelial-mesenchymal transition (EMT)[405]. EMT describes the process by which epithelial cells lose their polarity and cell-to-cell adhesion and become migratory through the basement membrane, whereupon they may differentiate into other tissue types and move from location to location. Once migrated, cells having undergone EMT may reverse the process to terminally differentiate in a new location by a process of mesenchymal-epithelial transition (MET)[406]. This process is crucial during embryological development and has been demonstrated to be critically related to healthy organogenesis and to congenital malformation[407]. EMT is also important in wound healing, where epidermal keratinocytes undergo an EMT-like process to re-epithelialise damaged tissues[408]. Beyond normal tissue remodelling, EMT has a pathological role in carcinogenesis; where dysplastic epithelial cells undergo invasive transformation to become invasive cancers[409]. Indeed, the definition of colorectal carcinoma (T1+), as separate from adenomas with dysplasia or carcinomas in situ (Tis: AJCC 0), is characterised by cellular invasion through the basement membrane and muscularis mucosae into the submucosa[30, 410]. Metastatic seeding may be considered as an analogous process to MET[411]. It is therefore important to gain an understanding of the processes that govern tumour cell migration and invasion so that the disease process may be better understood and combatted.

In *in vitro* studies of tumour behaviour, migration and invasion are considered as two separate entities defined and limited by the experimental design, although *in vivo* the two are considered to a large degree as part of the same EMT process[412]. *Migration* specifically refers to the ability of cells to move in two dimensions over a surface, which may be the inside of a plastic well or flask, an extracellular matrix (ECM) laid down in a plate, a glass slide, or any other flat substrate free of barriers. *Invasion* refers to the movement of cells through a matrix of substrate, necessitating remodelling of the substrate (such as an ECM) and modification of cellular architecture, cell-cell adhesion, polarity, and expression of proteins required for these processes. Invasion almost exclusively occurs in three dimensions, although there are some experiments that utilise a three-dimensional migration model where cells pass through pores in

a substrate without the need for ECM remodelling, and should be considered as a separate process. Although different methods of cellular motility may be found throughout biology, well-differentiated cancer cells migrate and invade primarily by mesenchymal migration in the manner of fibroblasts; relying on a strong interaction with the ECM, cytoskeletal contraction, and extensive spindle-like remodelling of the cell body[413]. A smaller proportion of cancer cells may migrate or invade by amoeboid movement, although this tends to be limited to melanoma and other irregularly migratory cancer types[414].

4.1.2.2 Wound Healing (Scratch) Assay

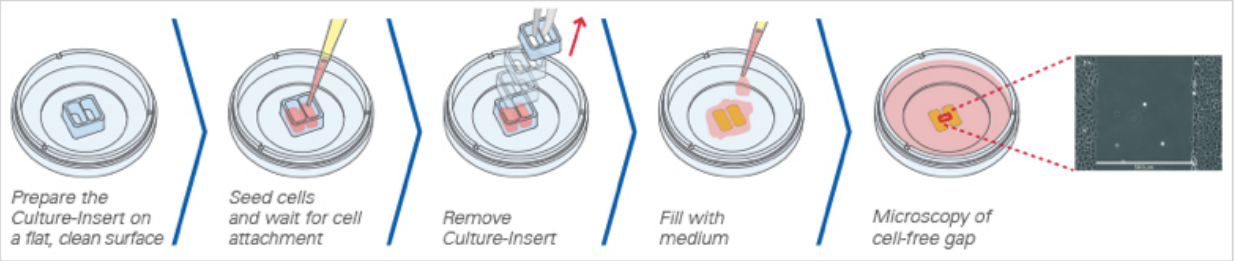
Scratch assays are a basic model of cell migration that utilises a "wounding" to a uniformly confluent sheet of cells growing on a two-dimensional substrate. The method can be as simple as performing a standard cell culture on a plastic well and scratching the confluent sheet with a pipette tip, although additional experimental complexity can be introduced by culturing cells on different substrates (collagen, ECM, etc), or by modifying other experimental variables. The time taken to restore confluence is usually taken as the primary end-point of the experiment, although as a measure of migration this is best estimated in short culture protocol (<24hrs) as longer culture times increase the proportion of confluence that is reached by proliferation rather than migration[415]. Generally, the technique is quick, cheap, and easily reproducible. Drawbacks include the unevenness of the scratch when performed with a pipette tip and the possibility that cells scratched from the sheet accumulate at the edge of the wound and may re-attach, artificially closing the wound. Investigators have attempted to control for these inaccuracies by electro-scratching the sheet of cells and assessing wound-closure by measuring electrical impedance, although these techniques have not been broadly adopted[416, 417].

4.1.2.3 Cell Exclusion Assay

Another method of performing a migration scratch-type wound healing assay is to construct a barrier to cell attachment at the time of seeding, a process referred to as micro-stencilling[418]. This may be performed by use of an electrical-fence, but more commonly by use of a gel-insert that is well-enough attached to the culture substrate that cells cannot adhere to the area below it following seeding. One of the more popular commercially available assays is the Ibidi Culture-Insert® system of re-usable silicone 2-well inserts with a 500μm cell-free exclusion zone (**Figure 4.1**)[419]. This, and other similar systems, has the advantage of producing a sharp

edged and reproducible cell-free zone, as well as minimising cell damage and reducing detached cells at the wound margin.

Figure 4.1 Ibidi Culture-Insert 2 Well System[419].

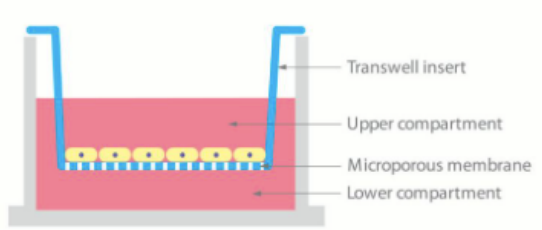


4.1.2.4 Boyden Chambers & Transwell® Systems

Both scratch and cell exclusion assays are only suitable for assessing cell migration as they are performed in two dimensions and do not involve active remodelling of the ECM. Boyden chambers, however, present the opportunity to assess cell migration and invasion in three dimensions by use of a suspended culture chamber with pores to allow cellular movement[420]. The base of the chamber can either be un-treated and inert to facilitate migration without invasion or be pre-treated with a layer of ECM through which cells must invade[421, 422]. Typically, a gradient between the top and bottom chambers is provided to induce cellular migration or invasion (such as a serum gradient or chemoattractant), and pore size chosen dependent on the size and characteristics of the cell line being studies. Typically pore sizes are between 3-12µm but must be smaller than the cells being examined to prevent "dropping". It is also important to consider the seeding density, port density, and two-dimensional migration speed of seeded cells, as low-density slow-to-migrate cells will increase the overall three-dimensional migration/invasion time during migration to a pore. Following culture, cells that have moved to the distant side of the membrane can either be fixed, stained, and counted directly (after removal of non-migrated cells from the top), or be detached and counted by a fluorescence counter following tagging. Boyden chamber systems have the advantages of being tailorable to different cell types and culture conditions, including co-culture, but require individual calibration for each cell type. They are generally more expensive than scratch or exclusion assays, but there are several commercially available models offering various potential benefits.

Transwell® systems exploit the physical set-up of the Boyden chamber but occlude the pores in the membrane by application of ECM, thus preventing the migration of non-invasive cells (**Figure 4.2**). Invading cells must degrade the ECM on the membrane before passing to the distant side of the membrane, induced by a gradient as previously discussed[423]. The most commonly used ECM is Matrigel® (Corning, New York, USA), a solubilised basement membrane extracted from Engbreth-Holm-Swarm (EHS) mouse sarcoma that has a high proportion of ECM proteins such as laminin, collagen IV, heparin sulfate proteoglycans, and others[424]. Matrigel is a good model for invasion through the ECM as it has characteristics that are highly similar to the protein and growth factor profile found in naturally occurring basement membrane[425-427]. As previously discussed, invasion of the basement membrane is the defining feature of invasive cancer, including CRC, and ECM remodelling has been observed in the invasive-front microenvironment[410, 428, 429]. Transwell® systems are available pre-treated with ECM in a variety of pore sizes, although modifications are feasible by purchasing individual components, and are very widely used in cancer research.

Figure 4.2. Transwell® System. An ECM such as Matrigel may be placed on top of the microporous membrane onto which cells are seeded[423].



4.1.2.5. Other Migration & Invasion Models

As well as the three models already described a number of other techniques for estimating cellular migration and invasion exist, although they are less popular. Fence assays utilise a reverse methodology to scratch and exclusion assays by introducing small areas of cells at confluence to larger areas of naïve substrate and observe outgrowth from the pool, rather than closure of a wound. Carrier-bead and spheroid migration assays take this principle further by introducing beads coated with confluent cells or spheroids to naïve substrate and observing outgrowth (akin to bacterial or fungal seeding on growth substrates for antimicrobial/fungal assessment). More complex invasion assays may be performed by constructing a platypus assay, where cells are sequentially sandwiched between two layers of ECM with an acellular

void in a proportion of the plate (created by exclusion), into which cells might invade laterally rather than through a porous membrane. Similarly, cells and spheroid may be introduced to larger blocks of gelatinised ECM and their free migration through or outward extension into the matrix may be observed like veins running through a block of blue cheese. Co-culture models of spheroid invasion (spheroid confrontation assay) may also be constructed in three-dimensional ECM blocks to introduce a further degree of three-dimensional and tissue microenvironmental complexity. Although the techniques and technology required for each of these assays is not advanced or expensive, they are time-consuming and prone to error, making them unsuitable for high-throughput screening or baseline biologic investigation, but may be appropriate in specific circumstances where a more elegant model is required[412].

4.1.3 *In Vitro* Demethylation in Colorectal Cancer Models

Epigenetic aberrations, including DNA hypermethylation, have been established as important factors in the molecular classification of CRC, although the pathogenic role is less well understood[118, 430]. *In vitro* studies of methylation in colorectal cancer have demonstrated that 10-day treatment with AZA at sub-cytotoxic doses can reactivate previously transcriptionally silenced genes, although the patterns of demethylation at specific promotor regions did not uniformly match gene reactivation[431]. However, in another study based on array analysis of 14,000 gene promotors in HCT116 cells, AZA treatment was demonstrated to produce specific and reproducible patterns of demethylation as well as conserved loci that were never demethylated[432]. A more recent study has found that demethylation of SW480 cells by AZA reactivates tumour suppressor NDN and potentially down-regulates *Wnt* signalling[433]. A further study examined the effects of AZA derivatives and 5-FU co-therapy on cell lines SW48 and HT-29, finding that there was potentially a synergistic effect with dual therapy[434].

4.1.4 *In Vivo* Demethylation in Colorectal Cancer Trials

The suggestion of synergy between AZA derivatives and other chemotherapy agents has led to a number of limited clinical trials in treatment-exhausted metastatic colorectal cancers, although results have so far been disappointing. A recently published phase II study of AZA with histone deacetylase inhibitor entinostat against multiply-treated metastatic colorectal cancer demonstrated no benefit[233]. A similarly disappointing outcome was found in a phase I/II trial of AZA with CAPOX in refractory CIMP-high metastatic colorectal cancer[232]. This study's methodology of determining CIMP-H was not well described and a tenuous focus on serum and stool vimentin hypermethylation as a proxy goes some way to undermining their

classification of CIMP-H, although their clinical outcomes were robust. However, another *in vitro* co-therapy study demonstrated that AZA acted synergistically with irinotecan to improve survival and improve tumour response in HCT116 CRC cell xenografted into mice, and has led to Phase I/II trials examining the efficacy of another DNMT inhibitor (Guadecitabine, SGI-110) with irinotecan in previously treated colorectal cancer, although results are yet to be reported[435]

4.2 Aims and Objectives

This chapter will examine the effects of demethylation on colorectal cell lines *in vitro*. Established methods of assessing locus-specific methylation (those already employed in **Chapter 3**) will be utilised for internal and external validity and consistency, as well as additional assessments of global methylation for broader perspective. Both a two-dimensional model of migration and three-dimensional models of invasion will be employed to determine the effects of demethylation in increasingly complex models that might provide an insight into and EMT/MET processes. Established (AZA) and putative (RRx-001) demethylating agents will be employed to investigate their comparative effects, following baseline assessments of their cellular cytotoxicity.

4.3 Methods

4.3.1 Cell Culture, Treatment, & Cytotoxicity

4.3.1.1 Cell Culture

Maintenance and control culture of DLD-1 cells was performed as outlined in **Section 2.3** and modified as described below for experimental purposes.

4.3.1.2 Treatment with Demethylating Agents

Both AZA and RRx-001 are stable in powdered form at -20°C and room temperature, respectively, but degrade when in solution. Although the rate of degradation for AZA is known (half-life dissolved in dH_2O or DMSO at room temperature at treatment concentrations (0.25-5.0 uM) is approximately 10-15hrs), the degradation of RRx-001 in solution and subsequent loss of biological activity is not known, although *in vitro* studies indicate it to be rapidly reactive with blood components and biologically effectively consumed almost immediately following initial administration[235, 239]. For the purposes of this study, a trial of the stability (in terms of biological efficacy) of each agent was undertaken by assessing cytotoxicity and demethylating ability of each agent following fresh preparation and storage at -80°C in stock concentration of 100 uM. Stored reagents were trialled alongside fresh-preparations, negative controls, and solvent controls to assess biological activity (molecular chemistry & pharmacokinetics of each compound was beyond the remit of this study). Both AZA and RRx-001 were found to be soluble in H_2O/DMSO at stock and working concentrations and stable at -80°C for at least one week, which was the maximum period these reagents were stored for when used in experimental models.

Each cell-culture experiment was conducted in multiple technical and biological replicates in order to assess and reduce sources of variation, and with negative, solvent, and where applicable positive controls (AZA acted as positive control for RRx-001)[436]. AZA and RRx-001 were presented across a dose-curve of 0.10, 0.25, 0.5, 1.0, 2.0, and 5.0 uM for initial screening of cytotoxicity and biological activity, and then the dose-curve rationalised for further experiments based on results. Doses were calculated and solutions of drug created by a serial dilution technique. This was to ensure the adequate mixing of drug in solution, and the accuracy and control between replicates of doses, especially at lower concentrations (versus direct pipetting of small doses).

4.3.1.3. Cytotoxicity

The number of cells observed in a population exposed to any particular treatment is the product of a number of factors, specifically; time, the starting population, rate of cell division (proliferation), and rate of cell death (natural apoptosis & toxicity). Each of these factors must be accounted for when assessing the effects of a chemical on a biological process so that effects on outcomes such as cell migration and invasion are not confounded by co-factors related to toxicity. As such, a baseline assessment of cytotoxicity and cytostasis must be made prior to experimetation[437]. The OECD Guidelines for the Testing of Chemicals (In Vitro Mammalian Cell Micronucleus Test) sets out the standards for assessing the cytotoxicity of a compound whether or not the investigator is employing actin polymerisation inhibitor cytochalasin B (cytoB); recommending that Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) be used to assess cytotoxicity and cytostasis when cytoB is not used[438]. Cytotoxicity should be assessed across a treatment gradient and in biological and technical replicate. The maximum cytotoxic effect used in biological modelling should be based upon a reduction of RPD of 55% +/- 5% at the maximum dose, although care should be taken when assessing outcomes at doses approaching this threshold. Additional tests examining endpoints such as cell integrity, apoptosis, necrosis, etc, can also be employed but should not substitute RPD or RICC[439, 440]. For the purposes of this research, a limit of 50% reduction in RPD will be set.

Assessment of RPD was made by culture of DLD-1 cells in control, solvent control, and treatment media and a dose-curve was employed to examine the cytotoxic effects of each compound. DLD-1 cells were grown until approximately 70% confluent according to standard culture conditions and then trypsinised and 5mls of cell suspension transferred to 25cm plastic cell culture flasks at a concentration of $3x10^4$ cells ml^{-1} (coulter counter assessment). Cells were allowed to adhere for a minimum of 8 and maximum of 12 hours (usually overnight) and then treated daily with AZA or RRx-001 (or control or solvent control) for a further 72hrs at 0.10, 0.25, 0.5, 1.0, 2.0, and 5.0 uM dose concentration. At the end of the 72hr period cells were trypsinised, washed, and re-suspended in 10ml PBS and counted on a haemocytometer or on the coulter counter.

RPD was calculated using the formula below.

$$\text{Population Doubling (PD)} = \frac{\text{Log (cell count after treatment / cell count in control)}}{\text{Log}^2}$$

$$\text{Relative Population Doubling (RPD)} = \frac{\text{Number of population doublings in treatment}}{\text{Number of population doublings in control}} \times 100$$

In addition to RPD, trypan blue, a commonly used stain that penetrates the membranes of dead but not viable cells, will be used for an additional assessment of cytotoxicity[441]. DLD-1 cells were cultured and treated as per the protocol for RPD testing. At the point at which cells were re-suspended in PBS for counting, cells were instead treated with 0.4% Trypan Blue solution (Sigma-Aldrich (Merck), USA) and incubated at room temperature for 5 minutes. Cells were then counted on a haemocytometer and a crude ratio of viable (unstained) cells versus dead cells (stained) was calculated.

4.3.2 Wound Healing Assay

An exclusion-zone assay was used as this provide the most easily reproducible and controllable method of assessing two-dimensional cell migration. The system employed was the Ibidi Culture-Insert 2 Well System as outlined above[419]. This provided a consistent 500μm "scratch" across which cells migrate on the base of a standard plastic cell culture well.

DLD-1 cells were grown until approximately 70% confluent according to standard culture conditions and then trypsinised and transferred to 25cm plastic cell culture flasks at a concentration of $3x10^4$ cells ml^{-1} (coulter counter assessment) and allowed to adhere for a minimum of 8 and maximum of 12 hours (usually overnight). Cells were then cultured for a further 72hrs in control, solvent control, or treatment (AZA or RRx-001) medium at a concentration based on cytotoxicity studies. Control or treatment media was refreshed daily.

After a full 72hrs of culture, treated (or control) cells were again trypsinised, washed, and diluted to a concentration of $5x10^5$ cells ml^{-1} in fresh culture medium. Ibidi cell culture inserts were applied to the base of 12-well culture plates and 70μl of suspended cells applied to each

side of the insert (either side of the exclusion zone). 700ml of suspended cells were applied to the well outside of the insert. After a period of 24hrs during which cell adherence to the base of the plate occurred, the insert was removed with sterilised forceps leaving a cell-free 500μm "scratch" in the adherent cell monolayer. A further 700ml of fresh culture media was added at this point to prevent dehydration during undisturbed culture during time-lapse microscopy.

12-well plates were then monitored by time-lapse light wide-field microscopy (frame capture once every 10 minutes) using the etaluma 560 Inverted Live-Cell Microscope platform. Time-to-convergence was measured by the clock starting when the Ibidi cell culture insert was removed and stopped when "first contact" was made between cells from either side of the cell-free exclusion zone. First contact was chosen as an endpoint as it favours cell migration as the predominant biological process (over cell proliferation), although wound-width, given by the formula below, was also employed an alternative endpoint (using Image J software)[442, 443]. Techniques to inhibit cell proliferation, such a mitomycin C, were not employed[444].

Formula for calculating Rate of Cell Migration.

$$R_M = \frac{W_i - W_f}{t}$$

R_M = Rate of cell migration (nm/h)
W_i = initial wound width (nm)
W_f = final wound width (nm)
t = duration of migration (hour)

In addition to the method set out above, an attempt was made to use the IN Cell Analyser (GE Healthcare) platform to facilitate high-throughput analysis of multiple wells in the 12-well plate, thus negating the limitation of the single-lens non-mobile platform of the etaluma system. Unfortunately, after several attempts, this was abandoned due to technical and physical constraints (programming/software errors, demand from other research groups performing time-sensitive experiments, physical location in another building thus requiring culture transfer). This did not affect the outcomes of the research, and in fact may have aided in increasing robustness through the necessity of performing multiple biological and technical replicates over an extended period.

4.3.3 Extracellular Matrix Invasion

A model of three-dimensional cellular invasion was constructed utilising the Corning (New York, USA) BioCoat™ Transwell® Boyden chamber system with a pre-loaded layer of Matrigel acting as the invasion matrix. This system provides a growth area of 0.3cm^2 of Matrigel ECM on top of a polyethylene terephthalate (PET) membrane perforated by 8-micron pores. Cells in the top chamber must therefore penetrate the EMC and translocate through the pores to the inferior aspect of the membrane in order to satisfy the definition for invasion. This system was chosen because of the likeness of the Matrigel ECM to the acellular components of the colorectal basement membrane and the ability of the DLD-1 cells to translocate through a pore of this size[425-427].

DLD-1 cells were cultures as per the protocol set out for wound healing assay (section 4.3.2) until the full period of 72hrs of treatment (or control) had been achieved. Invasion chambers were removed from storage at -20°C and allowed to come to room temperature and were then rehydrated in 1000µL of serum-free growth medium (500µL in each of the top and bottom of the wells/inserts) for 2 hours in standard incubator conditions. This rehydrating medium was then removed by pipetting, making sure not to damage the layer of Matrigel. A companion plate of invasion chambers consisting of PET membrane with 8micron pores but containing no ECM was also prepared.

Cells were trypsinised, washed, and made to a concentration of 3x10^5 cells ml^{-1} in serum-free medium. 750µL of fully composed medium (i.e. including serum) was added to the bottom compartment of each well and the invasion chambers (ECM & companion) carefully placed inside. 500µL of suspended cells was then placed in the upper portion of the invasion chambers atop the ECM and incubated for 24 hours. A variation to this protocol was also performed where 200µg ml^{-1} fibronectin was added to the bottom chamber to provide an additional chemoattractant (invasion gradient), as it has been shown to be an important factor in stimulation and regulating ECM remodelling and cellular invasion in healthy and cancerous tissue[445-447].

Following incubation, invasion chambers were removed from incubation wells and inverted to remove growth medium. The seeding-surface of the invasion membrane (top) was then wiped twice with a cotton bud to remove ECM and non-invading cells, as per manufacturers protocol. Invading cells on the under-surface of the invasion membrane were then fixed and stained by

sequential submersion in 100% methanol for two minutes and then crystal violet for two minutes, and then rinsed in dH_2O and left to air dry. Invading cells were then counted on a haemocytometer under light microscopy following removal of the invasion membrane from the chamber with a sharp scalpel.

Invasion was calculated as a percentage and index using the following formulae:

Percentage Invasion = (Mean number of cells invading through ECM / Mean number of cells invading through control membrane) x 100

Invasion Index = (% invasion for test (treated) cells / % invasion for control cells)

4.3.4 Assessment of Methylation Status

4.3.4.1 CIMP Status

Methylations status of control, solvent control, and treated DLD-1 cells was assessed by the same methodology of bisulfite-conversion and msPCR as set out in **Chapter 3** (**Sections 3.3.3, 3.3.4, 3.3.5**, and **3.3.6**). The only difference to these protocols was that DNA was harvested from cultured cells, negating the steps involving DNA extraction from FFPE slides. Cells were first trypsinised, washed with PBS twice, and then vortexed to pellet and the supernatant discarded. Cell pellets were then resuspended in proteinase K and Tissue and Cell Lysis Solution and the previously described protocol of the MasterPure Complete DNA and RNA purification kit (Epicentre, Illumina, Wisconsin, USA) followed. The quantity and quality of DNA was again measured by spectrophotometry and extracted DNA was stored at -20°C during active experimentation and archived at -80°C when not in use.

4.3.4.2 Global DNA Methylation

In addition to assessment by msPCR, global DNA methylation was also assessed by means of the MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) kit (Epigentek, New York, USA). In this assay, DNA harvested from DLD-1 cells is bound to pre-prepared wells containing antibodies with a high affinity to DNA and then capture-indicator antibodies specific to methylated DNA are added and the plate subjected to analysis by spectrophotometer, the proportion of methylated DNA being proportionate to the optical

density (OD). This method was chosen as it is widely used across the methylation literature to provide a broad and quantitative assessment of global DNA methylation[448, 449].

DNA was extracted from DLD-1 cells as set out previously, its quantity and quality assessed by spectrophotometry, and then diluted to 50ng μL^{-1} in DNA & RNA-free water. All reagents were prepared according to the manufacturers protocol (https://www.epigentek.com/docs/P-1030.pdf) and 100ng of DNA added to each sample well of the plate (see **Figure 4.3**). Negative controls and positive controls (both provided in the kit) were applied to the appropriate wells of the plate, including the 0.1% to 5.0% concentration gradient for the positive control that generates the standard curve for quantitative assessment. DNA and control solutions were gently swirled in the test wells and then the whole plate was covered with parafilm and incubated at 37°C for 60 minutes.

Detection Complex Solution (DCS) was then mixed according to supplied protocol and, after washing each well three times with 150µL of supplied washing buffer (WB), 50µL DCS was applied to each well. The plate was then re-covered with parafilm and left to incubate for 50 minutes at room temperature, after which the DCS was removed by pipetting and each well washed with 150µL WB a further five time. 100µL detection solution is then added to each well (vertical columns simultaneously) and the plate briefly agitated and then left for several minutes until the 5% PC wells turn dark blue, whereupon the stop solution is added in a similar fashion. Wells with a positive detection will then turn from blue to yellow after several minutes and the plate is read by spectrophotometry at 450nm within 15 minutes.

Percentage global DNA 5-mC can then be calculated using the formula shown below, following calculation of the slope from the standard curve. (OD, optical density; NC, negative control; S, amount of sample DNA in ng).

$$5\text{-}mC\% = \frac{Sample\ OD - NC\ OD}{Slope \times S} \times 100\%$$

Figure 4.3. Schematic of 96-well plate for MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit. (NC, negative control; PC, positive control).

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	1%PC	Sample 2	Sample 6	Sample 10	Sample 14
B	NC	1%PC	Sample 2	Sample 6	Sample 10	Sample 14
C	0.1%PC	2%PC	Sample 3	Sample 7	Sample 11	Sample 15
D	0.1%PC	2%PC	Sample 3	Sample 7	Sample 11	Sample 15
E	0.2%PC	5%PC	Sample 4	Sample 8	Sample 12	Sample 16
F	0.2%PC	5%PC	Sample 4	Sample 8	Sample 12	Sample 16
G	0.5%PC	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17
H	0.5%PC	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17

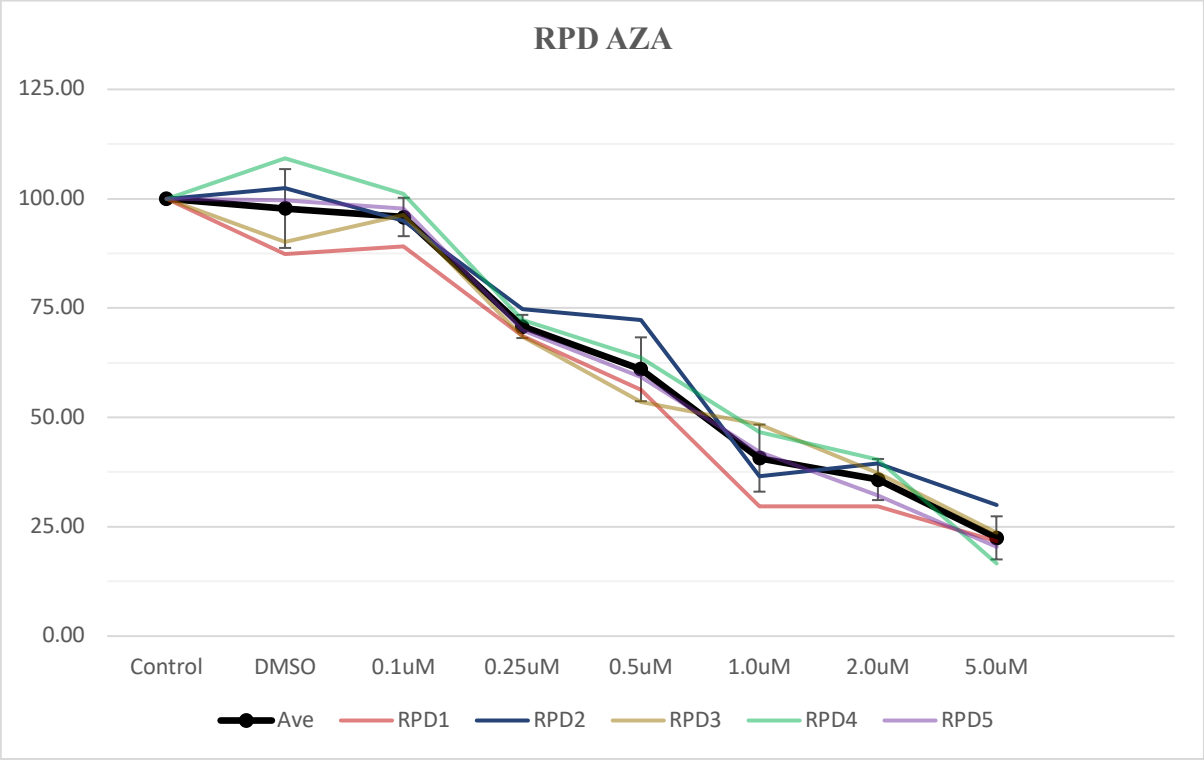
4.4 Results

4.4.1 Cytotoxicity

4.4.1.1 Azacytidine

AZA was applied to DLD-1 cells across a dose curve as described previously. At the end of the treatment period of 72hrs, cells were harvested and counted both by coulter counter and on a haemocytometer. Percentage RPD is shown in **Figure 4.4.1** across the dose curve, demonstrating a 50% reduction in RPD at between the 0.5uM and 1.0uM AZA doses. Averages of biologic replicates (typically 5) are represented by individual technical replicates RPD1-5, and an over-all average by the bold line (total replicates 25). Standard deviation (SD) at the 0.5uM dose is ±7.3% from a baseline of 61.0% RPD, and at no point on this series is ≥10.0%.

Figure 4.4.1 %RPD Dose Curve for Azacytidine. (Doses, where stated in uM, are for azacytidine (AZA), error bars indicated standard deviation).



Trypan blue analysis demonstrated that there was an increase in non-viable cells at AZA concentrations of greater than 1.0uM (≥30% non-viable), whereas concentrations below this level did not significantly affect the ratio of non-viable cells. The discrepancy between a significant effect of AZA on RPD (≥50% reduction at over 0.5uM) versus cell non-viability

(significant effects at ≥ 1.0uM) suggests biologic activity of AZA at lower concentrations that is not due to cell death.

4.4.1.2 RRx-001

RRx-001 was applied to DLD-1 cells across a dose curve as described previously. At the end of the 72hr treatment period, cells were harvested and counted both by coulter counter and on a haemocytometer. Percentage RPD is shown in **Figure 4.4.2** across the dose curve. Averages of biologic replicates (typically 5) are represented by individual technical replicates RPD1-5, and an over-all average by the bold line (total replicates 25). A 50% reduction in RPD at a dose of approximately the 1.0uM RRx-001 is demonstrated, however, standard deviation indicates that there is a high degree of variability of response to treatment at this dose (SD ±35.6% from 50% RPD), and thus an experimental dose of 0.5uM would be more appropriate (SD ±10.1% from 72.4% RPD).

Figure 4.4.2 %RPD Dose Curve for RRx-001. (Doses, where stated in uM, are for RRx-001, error bars indicate standard deviation).

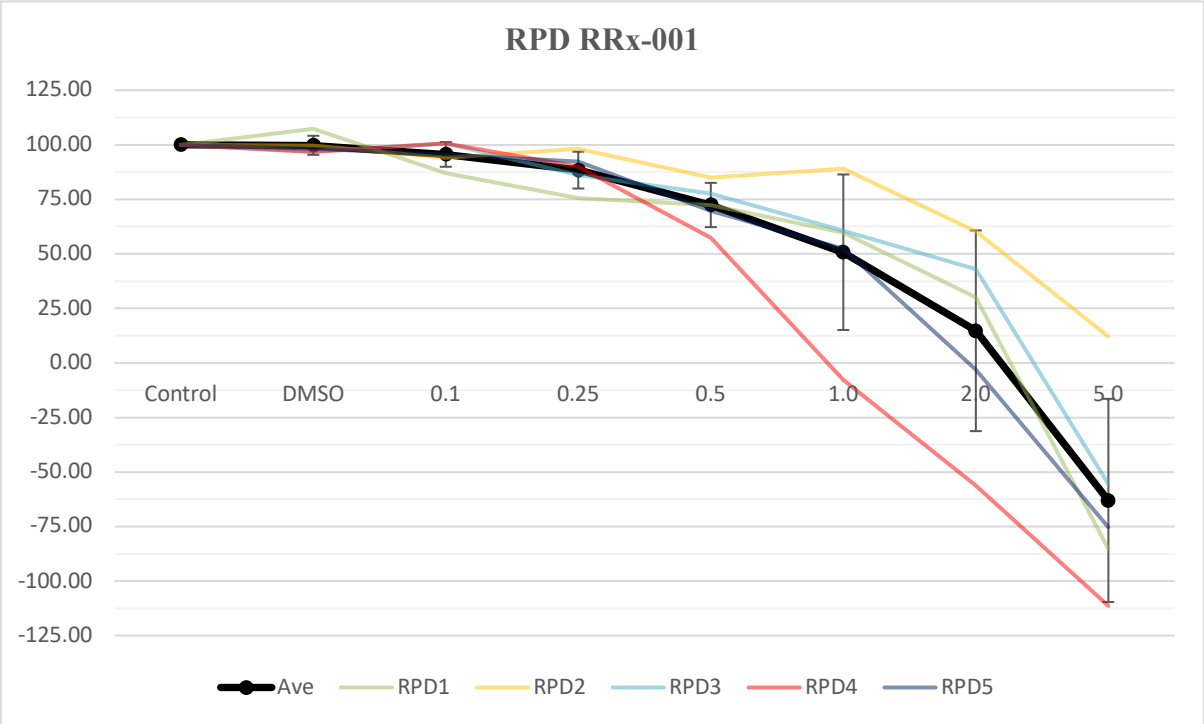


Figure **4.4.2** also indicates that at doses of ≥5.0uM, and more variably at 2.0uM, the RPD becomes negative, indicating a population decline compared to baseline seeding, and a highly cytotoxic effect of RRx-001. This was reflected in the trypan blue examination of RRx-001where there was a lower ratio of viable cells (≤70%) at doses of 1uM and above (<20% at 5.0uM). Viable cell ratios were however acceptable at doses up to 0.5uM RRx-001.

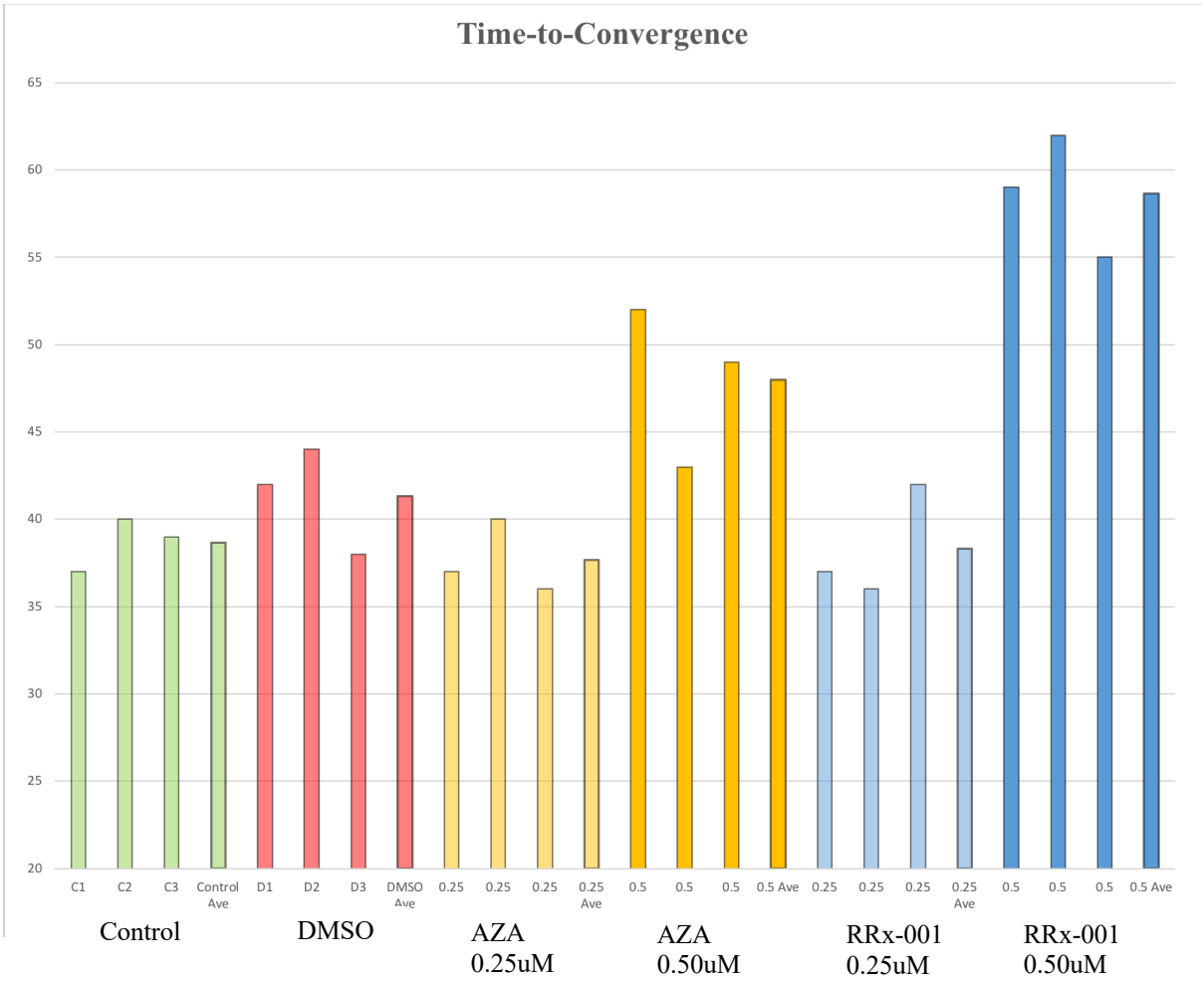
4.4.2 Two-Dimensional Migration (Scratch) Assay

Given the results of the cytotoxicity assays (RPD and trypan blue), a maximum experimental dose of 0.5uM over a 72hr exposure period (daily treatments) of both AZA and RRx-001 was employed as a baseline for wound healing and invasion assays.

Using the Ibidi culture system, untreated control and solvent control (DMSO) exposed DLD-1 cells took a mean time of 38 hours (SD ±1.5) and 41 hours (SD ±3.0), respectively, to converge (reach first contact) across the 500μm cell-free zone. Prior treatment for three days with either 0.25uM or 0.5uM AZA resulted in a mean convergence time of 37.5 (SD ±2.0) and 48.0 (SD ±4.5) hours, respectively. Prior treatment for three days with either 0.25uM or 0.5uM RRx-001 resulted in a mean convergence time of 38.5 (SD ±3.0) and 58.5 (SD ±4.5) hours, respectively. The difference of the means between the control and 0.5uM treatments with both AZA and RRx-001 were highly significant based on students t-test ($p<0.001$), but not for DMSO or the lower doses of 0.25uM of either compound. These results are shown in **Figure 4.4.3**.

Figure 4.4.4 demonstrates the process of cell migration across the void using the Ibidi cell system, and the measured endpoints for illustrative purposes. **Image a.** demonstrates the starting position of the cells in culture at timepoint 0, with the gel insert only just removed. **Image b.** demonstrates the point of first convergence PoC, where cells from either side of the void make physical contact and stop the clock (time to convergence, TtC). **Image c**. demonstrates a greater degree of convergence although complete exclusion of the void is yet to occur. Images **a.** and **b.** have also been marked-up to demonstrate the area that remains to be filled-in by cells that forms the final width metric that could be used to calculate the Rate of Cell Migration, although this was only applied when cells reached PoC as demonstrated in **image b** as this is the least subjective time-point for assessing convergence. **Image d.** illustrates almost complete occlusion of the void and proliferative overgrowth away from the original cell-free zone.

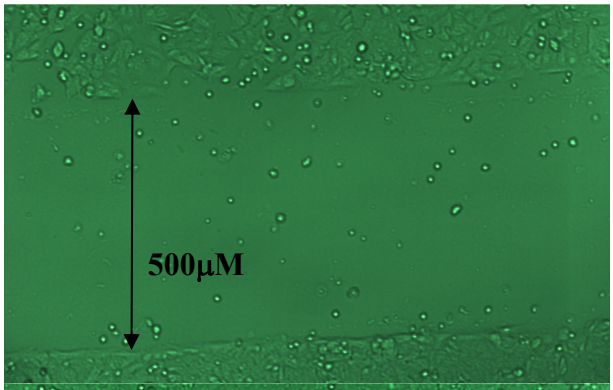
Figure 4.4.3 Time-to-Convergence (first contact) for DLD-1 cells across a 500µm cell-free zone.



	Mean Time-to-Convergence (TtC)	Range TtC	Standard Deviation	p (Students-t)
Control	38.5 hrs	37.0 – 40.0 hrs	±1.5 hrs	-
Solvent Control (DMSO)	41.5 hrs	38.0 – 44.0 hrs	±3.0 hrs	NS
AZA 0.25uM	37.5 hrs	36.5 – 40.5 hrs	±2.0 hrs	NS
AZA 0.50uM	48.0 hrs	43.0 – 52.0 hrs	±4.5 hrs	<0.001
RRx-001 0.25uM	38.5 hrs	36.0 – 42.5 hrs	±3.0 hrs	NS
RRx-001 0.50uM	58.5 hrs	55.0 – 62.5 hrs	±3.5 hrs	<0.001

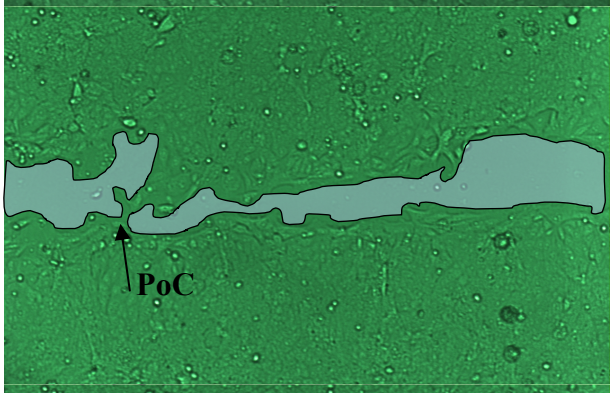
Figure 4.4.4 Etaluma light microscopy images demonstrating stages in cell migration during the wound healing (scratch) assay.

a.



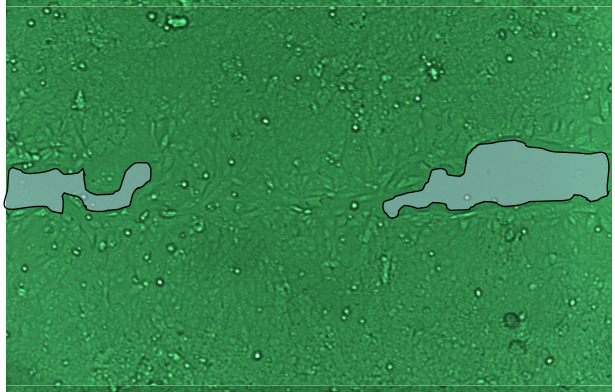
DLD-1 cells at time point 0 immediately after the gel insert has been removed. Note the 500µM cell-free void (scratch) between the relatively confluent cell fronts.

b.



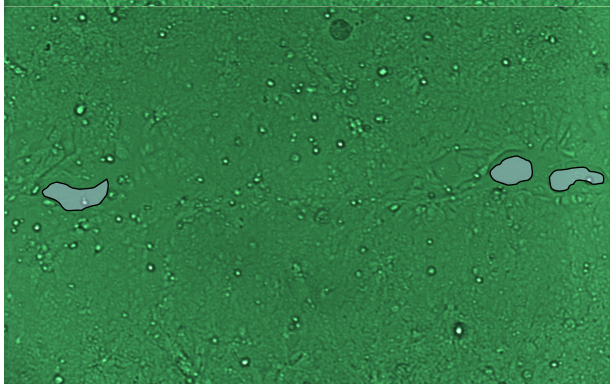
DLD-1 cells at the point of convergence (PoC), representing the 'clock-stop' for Time to Convergence. Note cell-free shaded area that forms the basis of the width (area) metric.

c.



DLD-1 cells beyond the PoC but with some cell-free void remaining. Note the cell morphology along the convergent zone; elongated cells have bridged the void, suggesting a cell-migratory process, rather than proliferative.

d.

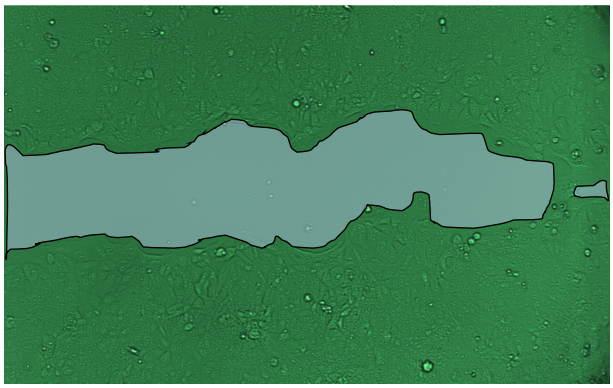


DLD-1 cells at beyond PoC. There are still a few cell-free voids but >70% confluence has been reached and the cells behind the convergent zone are crowded, demonstrating a proliferative process away from the original cell-free zone.

The Rate of Cell Migration metric R_m was found to be unreliable. Firstly, for the formula set out in **Section 4.3.2**, R_m is defined as the *initial width*, minus the *residual width*, divided by *time*. Employing the PoC definition as described (the *residual width* being 0), the only independent variable is *time*, and therefore the metric R_m = 500µM / TtC and is not a better discriminator over TtC alone, when examining the effects of AZA and RRx-001 on migration. Secondly, if the metric R_m is modified to replace *initial width* and *residual width* with *initial area* and *residual area*, as demonstrated by the shaded areas on **Figure 4.4.5 a. – c.**, no advantage was found over TtC alone, nor was a statistically significant difference in mean cell-free area at PoC evident.

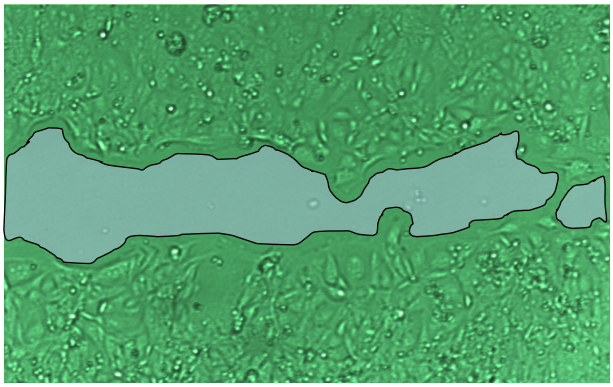
Figure 4.4.5 Examples of PoC and residual cell-free areas for Control, AZA, and RRx-001.

a.



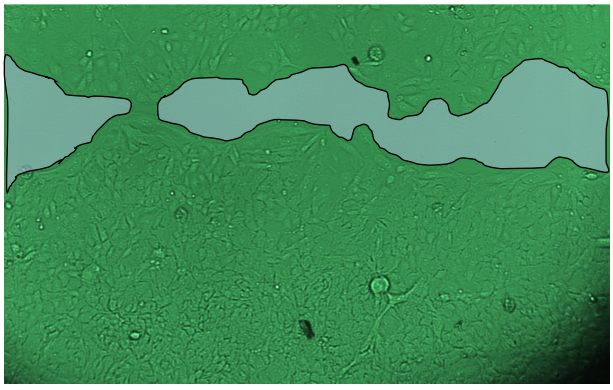
Control DLD-1 cells at PoC. The shaded cell-free area is measured at 154,000µM^2.

b.



DLD-1 cells exposed to 0.5uM AZA at PoC. The shaded cell-free area is measured at 149,000µM^2. Students-t test for significant difference between mean area in treated vs control cells was non-significant.

c.



DLD-1 cells exposed to 0.5uM RRx-001 at PoC. The shaded cell-free area is measured at 145,500µM^2. Students-t test for significant difference between mean area in treated vs control cells was non-significant.

4.4.3 Three-Dimensional Invasion Assay

Given the results of the cytotoxicity assay, a maximum experimental dose of 0.5uM of both AZA and RRx-001 was employed in invasion assays over a 72hr exposure period with a daily dosing regime.

After 24 hours incubation there was no difference in the mean percentage invasion (PI) between control and solvent control (DMSO) exposed DLD-1 cells (100% and 97-98%, respectively), with a mean invasion index (II) of 77 and 75, respectively. Prior treatment for three days with either 0.25uM or 0.5uM AZA resulted in a mean PI of 50.0% (SD ±3.4%) and 31.1% (SD ±3.1%), and II of 42 and 27, respectively. Prior treatment for three days with either 0.25uM or 0.5uM RRx-001 resulted in a mean PI of 49.5% (SD ±2.6%) and 33.10% (SD ±2.4%), and II of 41 and 28.5, respectively. The difference between the mean PI of the control DLD-1 and DLD-1 exposed to either 0.25uM or 0.5uM treatments with either AZA and RRx-001 were highly significant based on students t-test ($p<0.001$), but not for DMSO. The addition of 200μg ml^{-1} fibronectin, an established chemoattractant, resulted in an increase in the baseline percentage invasion, PI, at control and solvent control (mean increase PI =11) and was maintained across the experimental arms with both compound at all doses. This increase did not however cause a significant change to the invasion index, II, as it affected controls and experimental arms equally. These results are shown in **Figure 4.4.6** and **Figure 4.4.7**.

Figure 4.4.6. Light microscopy demonstrating haemocytometer counts of invading DLD-1 cells on the underside of the invasion well. Note the 8micron pores in the film. Cells have been fixed with 100% methanol and crystal violet. Image **a** – control; **b** – control + fibronectin; **c** – 0.25uM AZA; **d** – 0.50uM AZA.

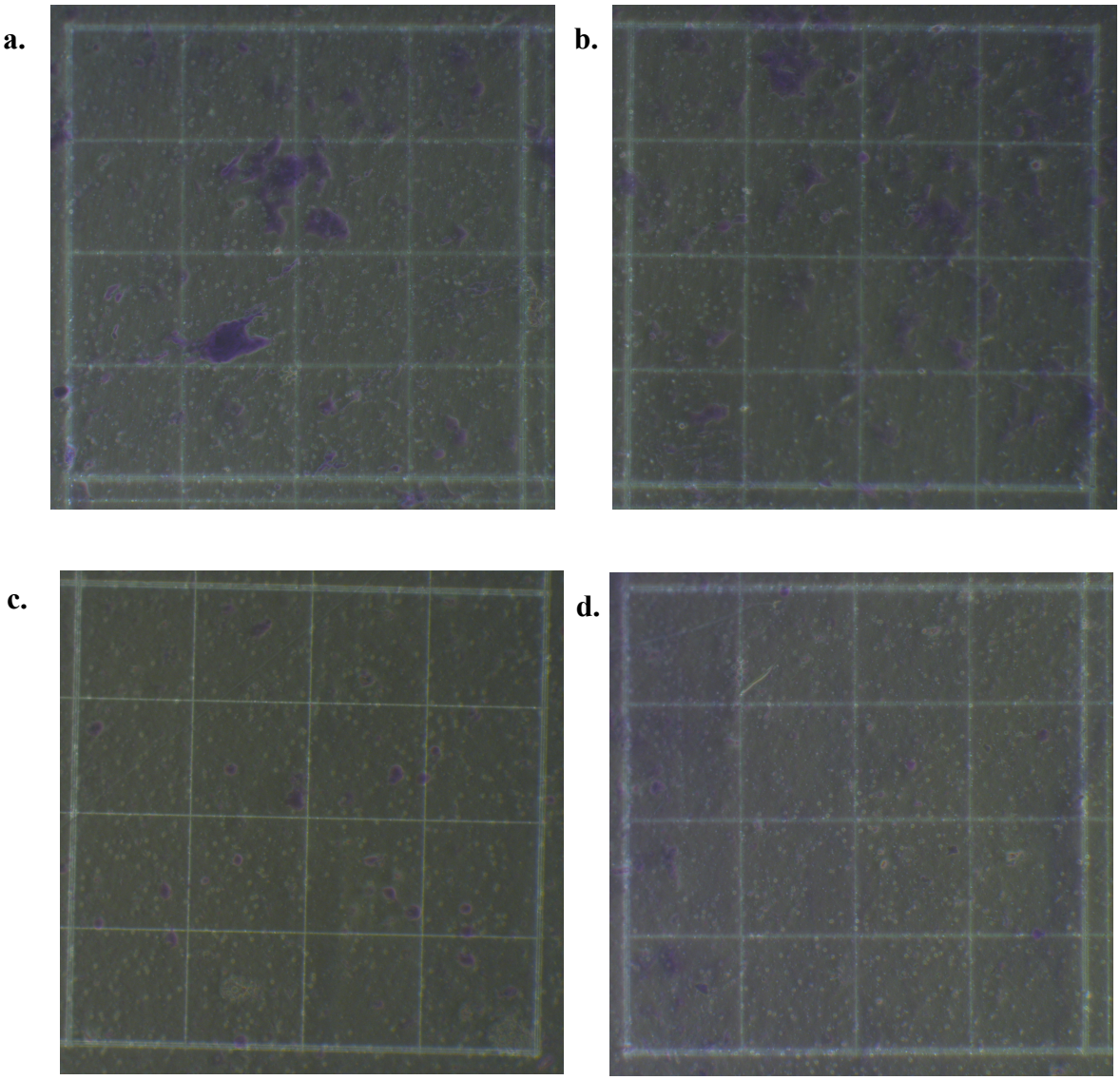
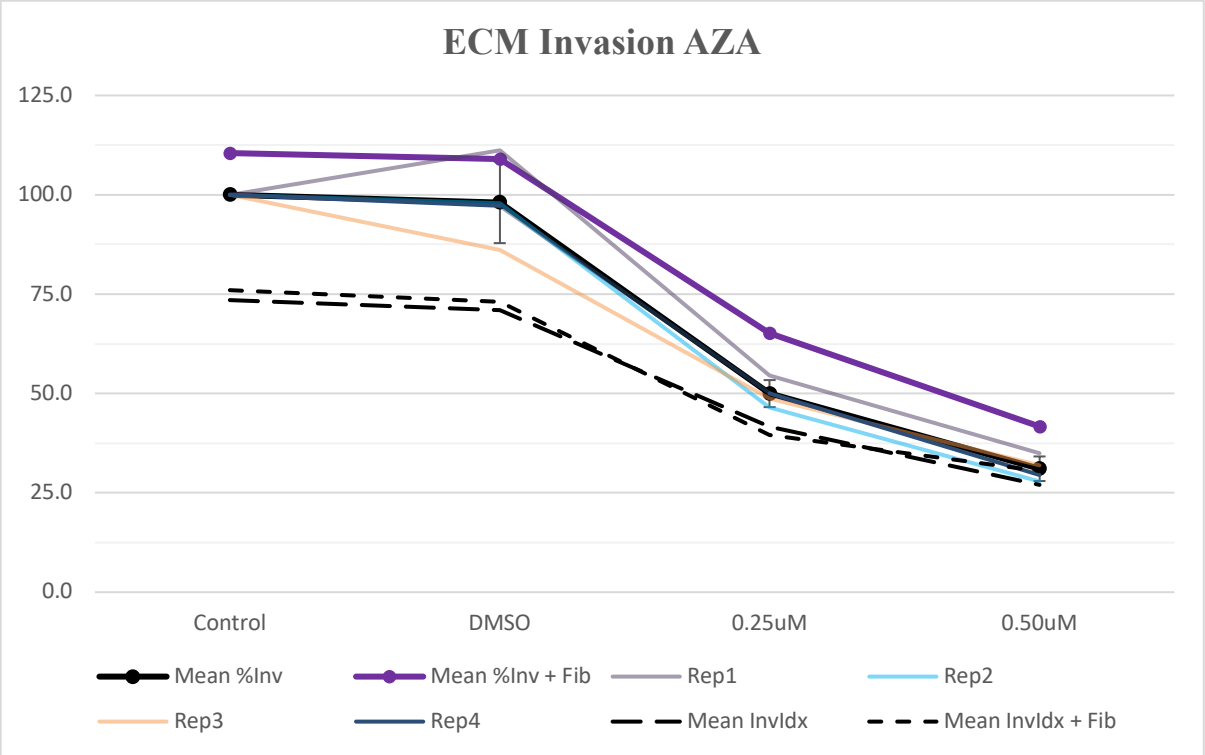
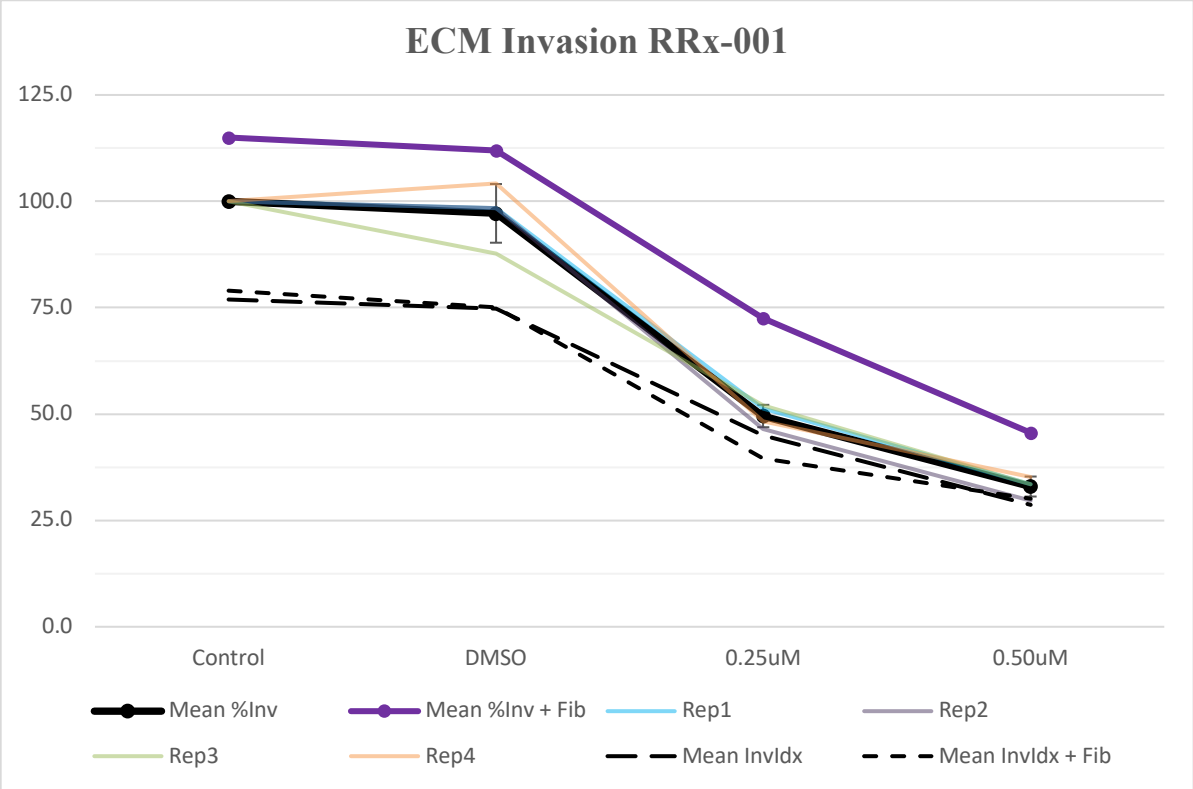


Figure 4.4.7 DLD-1 invasion through Matrigel ECM expressed as Percentage Invasion (PI) and Invasion Index (II).

a. DLD-1 PI & II versus AZA



b. DLD-1 PI & II versus RRx-001



4.4.4. Demethylating Effects of Azacytidine and RRx-001

4.4.4.1 CIMP Status

Methylation status of control, solvent control, and treated DLD-1 cells was assessed by the same methodology of bisulfite-conversion and msPCR as set out in **Chapter 3** (**Sections 3.3.3, 3.3.4, 3.3.5**, and **3.3.6**). Cells were first cultured for three days and exposed to AZA 0.50uM or RRx-001 0.50uM according to the protocol employed for cytotoxicity and migration / invasion assays in **Section 4.3.1** onwards.

The baseline methylation status of DLD-1 cells is pan-methylated at each of the loci employed in the experimental panel, evidenced by strong amplification of DNA by the methylated-specific primers in the control and solvent control datasets. There was, however, a minor degree of amplification of non-methylated DNA for both SOCS and IGFBP, although this did not reach the 10% image density saturation (Gel Doc XR (Bio-Rad) system, Quantity One software) threshold for non-anomaly when analysed against the control bands.

Following exposure to 0.50uM AZA, DLD-1 cell DNA demonstrated significant de-novo demethylation at five out of eight loci; MINT, HAND, ADAMTS1, NEUROG, and THBD. This effect, however, was not universal across the entire cell population, as DNA was extracted which also amplified against the methylated primers, although non-methylated amplification was well above the 10% threshold, indicating substantial demethylation. The previous patterns of methylation for the other three loci (hMLH, SOCS, and IGFBP) remained unchanged.

Following exposure to 0.50uM RRx-001, DLD-1 cell DNA demonstrated significant de-novo demethylation at four out of eight loci; MINT, HAND, NEUROG, and THBD. Again, demethylation at these loci was incomplete across the extracted DNA and therefore the cell population, as DNA was also amplified against methylated primers at these loci. Subjectively, the bands representing amplification of unmethylated DNA at the HAND, NEUROG, and THBD loci were weaker than that observed following exposure to AZA, suggesting a lesser degree of demethylation. The methods used in the assay were not, however, quantitative, and thus this result should be considered as qualitative only. The previous patterns of methylation for the other three loci (hMLH, SOCS, ADAMTS1, and IGFBP) remained unchanged.

According to the epigenotype classification described in **Section 1.3.6**, DLD-1 cells are CIMP-H at baseline and remain so despite demethylation (with either AZA or RRx-001) of MINT and the Group 2 Markers[185]. The above results are summarised in **Table 4.1**.

Table 4.1 Methylation status of selected individual genes and CIMP Status of DLD-1 cells after exposure to AZA and RRx-001. M = methylated, U = unmethylated, + = strong, - = weak, H = CIMP High.

	hMLH	**MINT**	**SOCS**	**HAND**	**ADAMTS 1**	**NEUROG**	**IGFBP**	**THD**	**CIMP Status**
Control	M	M	M	M	M	M	M	M	H
AZA	M	U	M	U-	U	U	M	U	H
RRx-001	M	U+	M	U-	M	U-	M	U-	H

4.4.4.2 Global Methylation

The MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) kit measured at 450nm was used to assess global DNA methylation (global 5-mC) in control DLD-1 cells and in cells exposed to AZA and RRx-001. As this method allows global quantitative assessment of DNA methylation, a dose curve methodology was applied.

A standard curve for analysis of methylation at ≤1% global 5-mC was constructed using the controls provided in the kit and a linear regression model, yielding an $R^2 = 0.8136$, which was improved to $R^2 = 0.9542$ if the upper limit was set at ≤0.5% global 5-mC, and delivering a slope of $b = 0.414$ (**Figure 4.4.8 a.**). This is in accordance with the manufacturer's recommendation for the use of 4 data points (0%, 0.1%, 0.2%, and 0.5%) to attain a slope at the most linear section of the curve.

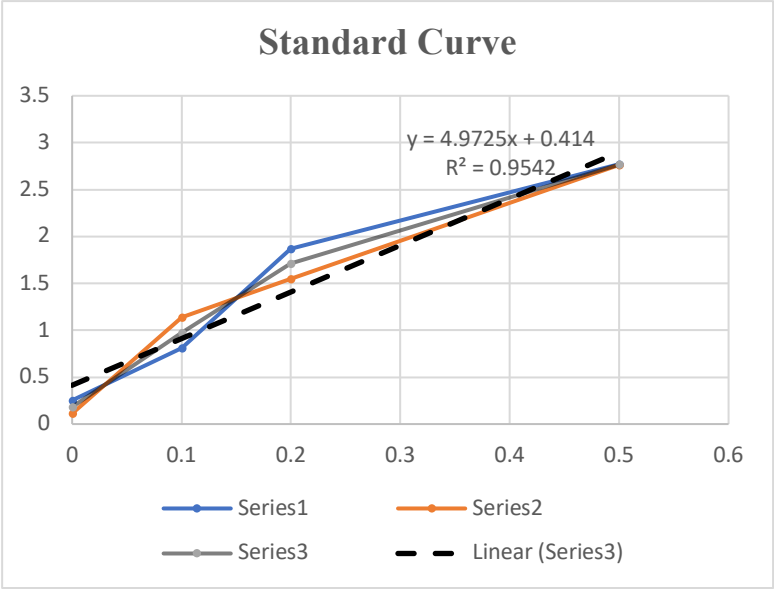
Both AZA and RRx-001 effectively demethylated DLD-1 cells, as demonstrated in **Figure 4.4.8 b**. The total percentage of methylated cytosines within DNA of control DLD-1 cells was 0.05676% (0.27% global cytosine - GC), which fell to 0.00008% (0.0004% GC) in cells treated with 0.5uM AZA and to 0.02093% (0.0996% GC) in cell treated with 0.50uM RRx-001. Treatment with AZA demonstrated a precipitous demethylation of cytosine, even at very low

doses, whereas the demethylation demonstrated by RRx-001 was dose-dependent up to 1.0uM. Above doses of >1.0uM of both agents a paradoxical rebound in global 5-mC was observed.

Figure 4.4.9 also demonstrates the global demethylation produced by both AZA and RRx-001 at a macro level by showing the ELISA plate colorimetric output. Note the both the precipitous decline in the proportion of 5-mC with AZA, and the dose dependent relationship with RRx-001, as well as the paradoxical increase in 5-mC at the 1.5uM dose of each agent.

Figure 4.4.8 a. Standard curve for analysis of global 5-mC ELISA & **b.** global methylation in DLD-1 cells, expressed as percentage of methylated cytosine.

a.



b.

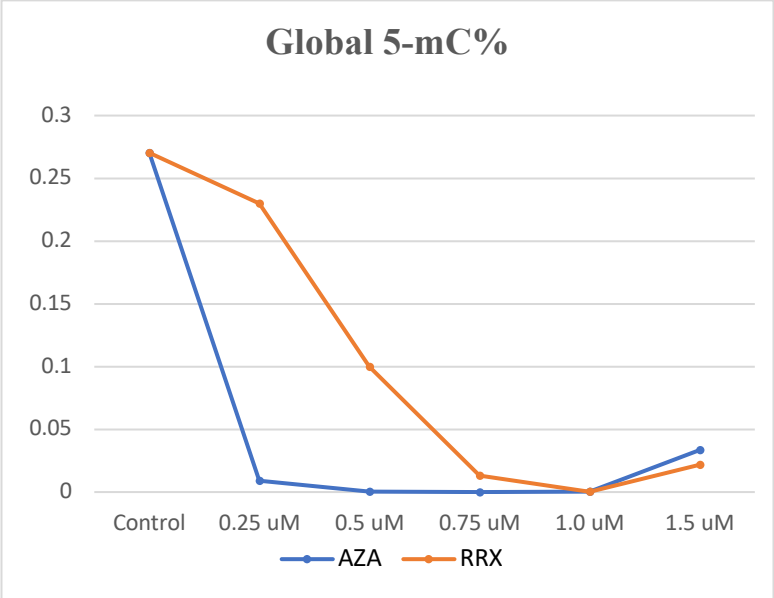
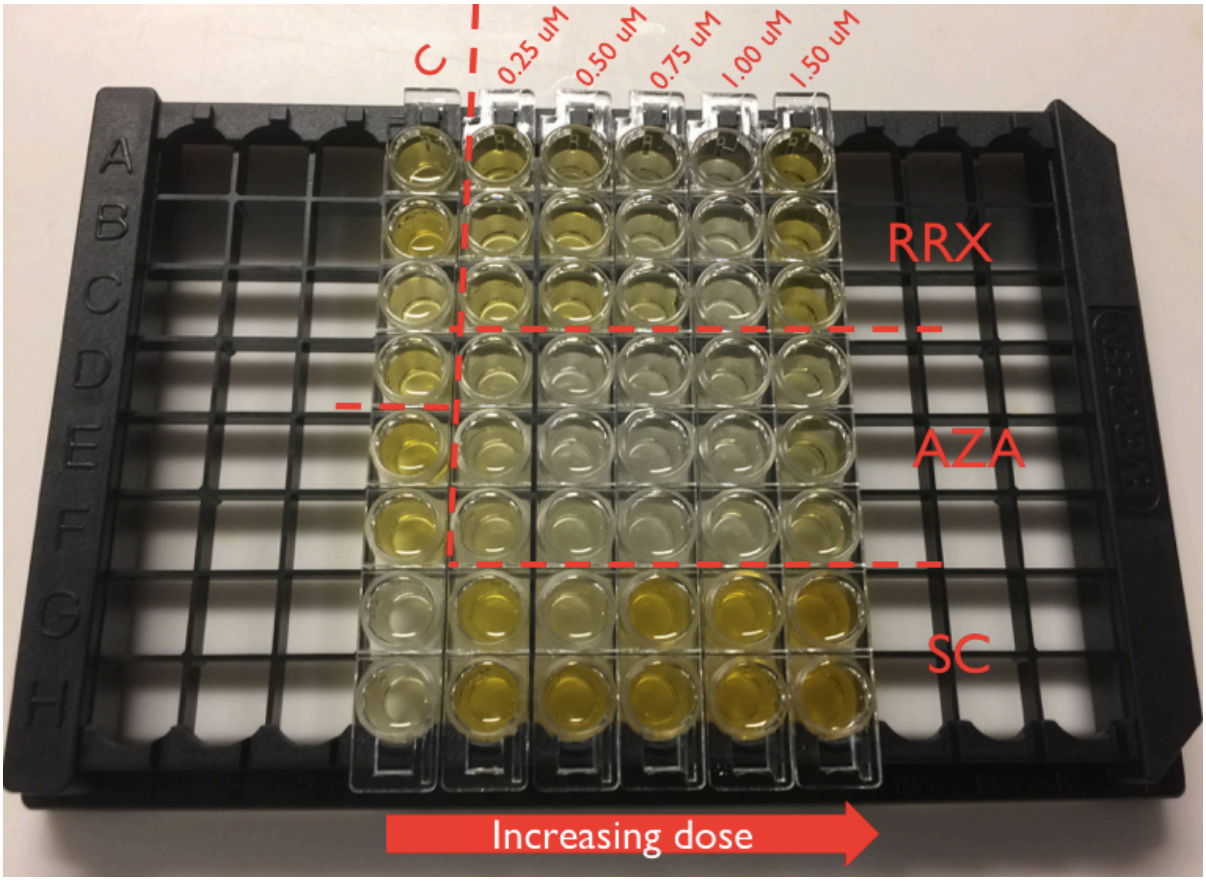


Figure 4.4.9 Resolved MethylFlash Global DNA Methylation (5-mC) ELISA plate demonstrating differential demethylation with AZA and RRx-001. Yellow = methylation.



4.5 Discussion

This chapter's aims were to examine the effects of demethylation on a colorectal cell line *in vitro*, and to set parameters for the further investigation of demethylating agents in a model of colorectal cancer epithelial-mesenchymal and mesenchymal-epithelial transition (EMT/MET). To this end, the first element of this chapter was to establish the baseline cytotoxicity of AZA and RRx-001 in DLD-1 cells so that any effects observed in subsequent experiments cannot be confounded by toxicity. The second element of this chapter was to examine the *in vitro* biologic effects of AZA and RRx-001 at sub-cytotoxic doses on DLD-1 cells in models of EMT & MET. The third element was to establish the demethylating effects of AZA and RRx-001 at sub-cytotoxic and biologically effective doses, both in terms of the locus-specific methylation status employed by the Kaneda & Yagi CIMP panel, and global methylation[185]. By these means the overarching aim was to establish a three-way-look at the investigation of an association between the methylation status of DLD-1 cells the biologic activity that is dependent on their methylation status.

4.5.1 Cytotoxicity of Azacytidine and RRx-001

An appropriate upper-limit for cytotoxicity is important when selecting doses of potentially biologically active or mutagenic compounds since excessive cytotoxicity is a well-established source of misleading positive results[439, 450]. Population-doubling (RPD) is advocated by many authors as a reliable method of excluding cytotoxicity as a result of disruption to off-target cellular physiological processes and is supported by the OECD and other regulatory authorities[437, 438, 451]. Based on this guidance an upper limit of 55±5% RPD was set as a cut-off for both AZA and RRx-001; doses above this being considered cytotoxic and doses below being considered non-cytotoxic. Exposure times were informed partly on the basis of prior experimentation within our own department, and also in accordance with other authors' protocols[452]. For both compounds, 55±5% RPD was reached between 0.50uM and 1.00uM, with 0.50uM of AZA and RRx-001 being considered as a reliably sub-cytotoxic dose (see **Section 4.4.1** for full results). These finding are broadly in keeping with those previously published by other authors investigating the *in vitro* effects of AZA on a variety of cell lines, although both higher and lower concentration tolerances have been reported[209, 453]. Interestingly, Juttermann and colleagues suggest that the cytotoxic effects of AZA observed at higher concentrations (≥0.50uM in their investigation) are caused by an irreversible covalent trapping of DNMT to DNA, rather than the effects of demethylation itself, underlining the significance of establishing sub-cytotoxicity when investigating the specific effects of

demethyltion[213]. As RRx-001 is a novel compound, there is little published evidence on its biologic effects and cytotoxicity *in vitro*, although doses of between 0.5uM and 5.0uM have been found to be sub-cytotoxic (and demethylating) in squamous cell carcinoma (SSC VII) cells[243]. Furthermore, the same study examined the comparative global demethylating effects of RRx-001 against AZA at doses of between 0.5uM and 2.0uM, finding a comparative epigenetic effect. This is consistent with another *in vitro* study, where RRx-001 was found to modulate DNMT1 and DNMT3a activity by increasing free oxygen and nitrogen radicals, reducing the abundance of methylation substrates and thus causing demethylation[454]. The establishment of sub-cytotoxic doses of both AZA and RRx-001 at ≤0.50uM concentrations was also supported by the favourable results of the trypan blue assays. Furthermore, as RPD is a more sensitive measure of early toxicity, effects observed inviable cells following protracted treatment are likely to represent biologic effects rather than toxicity.

4.5.2. The *in vitro* biologic effects of AZA and RRx-001

Wound healing (scratch) and Boyden chamber-type assays are established methods of investigating cell migration and invasion[419, 420]. Increasing elements of complexity may be added to the basic experimental design to create cellular environments more comparable to those that are key in the EMT and MET processes[422, 423]. The results of the scratch assay (**Section 4.4.2**) demonstrated that, at sub-cytotoxic doses, both AZA and RRx-001 significantly increased the time taken for DLD-1 cell to migrate across the cell-free zone ($p<0.001$ for both compounds). Similarly, AZA and RRx-001 at the same sub-cytotoxic doses reduced the ability of DLD-1 cells to invade through the ECM and migrate through the porous membrane of the Transwell® Boyden chamber system ($p<0.001$ for both compounds) (**Section 4.4.3**). These results suggest that both compounds reduce the migratory and invasive propensity of DLD-1 cells in *in vitro* models of EMT and MET, therefore suggesting that hyper-methylation could increase motility and invasion in the carcinogenic process.

The results of this section are in keeping with the findings of other investigators, who have reported that other colorectal cell lines (HCT-116) were less able to migrate across and invade through a control and ECM treated Transwell system, respectively, following treatment with AZA[452]. Another similar study, this time employing different colorectal cancer cell lines (SW480 and SW620) and decitabine (an AZA derivative), also demonstrated a reduced propensity for CRC cell to migrate and invade following treatment with a demethylating agent[455]. Despite the different cell types and demethylating agents used in these studies, the

alignment with the findings from this investigation suggest a consistency in the application of demethylating agents to CRC cell lines and the reduction in the ability of cells to invade. This is particularly interesting when considering the EMT/MET process, especially in the case of SW620 which is a lymph node metastasis derived colorectal cancer cell line.

Similar findings have also been reported for other tumour types, including oesophageal squamous cell carcinomas and adenocarcinomas, renal cell carcinomas, gastric carcinomas, and laryngeal squamous carcinomas[456-459]. In a study of multiple oesophageal tumour cell types, treatment with AZA (and/or decitabine) resulted in a reduced ability of tumour cells to migrate and invade, but only when dual therapy with other epigenetic modifiers was applied; specifically, histone deacetylases (HDACs)[456]. These findings were echoed in another study examining the effects of dual treatment with the HDAC valproic acid in conjunction with AZA on renal cell carcinomas (786-O and 769-P) *in vitro*[457]. These studies suggest that there is a synergistic effect between demethylating agents such as AZA with other epigenetic modifiers, and that the biological effects may only be observed when the two are applied in tandem, counter to the findings in this chapter. However, as well as the previously discussed studies on colorectal cancer cell lines, other authors have also found that monotherapy with AZA effectively reduces the ability of cancer cell lines to migrate and invade. This is demonstrated by the study on gastric cancer (AGS cells) by Zuo and colleagues, who demonstrated reduced migration and invasion with AZA monotherapy[458]. Lui also found that AZA monotherapy reduced the ability of laryngeal squamous cells (HEp-2) to migrate and invade, based on scratch and Transwell assays[459].

No previous study has examined the biologic effects of RRx-001 in terms of the propensity of treated cells to migrate or invade. The results in this chapter indicate that, at sub-cytotoxic doses, RRx-001 significantly reduces the ability of DLD-1 cells to migrate in two-dimensions in a wound healing assay, and to invade in three-dimensions through the ECM components of Matrigel in a Transwell Boyden-chamber assay. This was demonstrated by the reduced percentage invasion (PI) and invasion index (II) with increasing doses of RRx-001 when compared to control and was statistically significant ($p<0.001$). The reduction in invasion was comparable to that observed with the same doses of AZA. The addition of fibronectin to the bottom chamber increased the percentage invasion by 10-15% at each dose-point for both AZA and RRx-001 but did not increase the II as it also proportionately increased the denominator.

4.5.3. Assessment of Methylation Status

At baseline, DLD-1 cell were found to be CIMP-high based upon the two-panel classification system described in by Kaneda and Yagi, and summarised in **Section 1.3.6**[185]. Each of the eight loci was found to be methylated; each demonstrating strong amplification at the specific CpG islands against the methylation specific-primers. A minor degree of amplification was evident for both SOCS and IGFBP, although this did not reach the 10% threshold for significant heterogeneity.

Following treatment with AZA at 0.50uM concentration, DLD-1 demonstrated significant but incomplete demethylation of five out of eight loci; MINT, HAND, ADAMTS1, NEUROG, and THBD. Although incomplete, demethylation of these loci was significant as the demethylated bands for these sites was stronger than the methylated bands, which is converse to the baseline methylation status. The finding that AZA is an effective demethylator of human DNA is not unexpected, as it is a well-established demethylating agent with widespread laboratory and some clinical applications (see **Section 2.2.3** of General Methodology). The previous patterns of methylation for the other three loci (hMLH, SOCS, and IGFBP) remained unchanged. Despite demethylation at five of the eight loci, the CIMP status of DLD-1 cells exposed to AZA remains CIMP-high, as two of the Group 1 markers remain methylated (hMLH and SOCS).

AZA also produced a dramatic decline in global 5-mC, as demonstrated by the ELISA. At 0.50uM dose of AZA, global 5-mC was reduced by a factor of over 700, from 0.05676% of global DNA to 0.00008%. The figure of just over a twentieth of 1% for baseline 5-mC is objectively very low, with expected levels of methylated cytosine in human DNA typically being approximately 1%, dependent on the tissue[460, 461]. Indeed, Donoghue reports 62% methylation at CCGG sites throughout DLD-1 DNA, suggesting a much higher percentage of global 5-mC[462]. These findings do not however take account of the stochastic variations in DNA methylation observed in cancer cells, or the acknowledged pan-hypomethylation across the entire epigenome despite the locus-specific hypermethylation observed in CIMP-high tumours[463]. Additionally, although there are multiple methods of determining global DNA methylation, the ELISA-based method employed in this chapter is considered reliable and validated against human and non-human DNA and in a variety of disease states, including cancers[448, 464, 465]. Despite the low absolute figures, what is clear from the global 5-mC ELISA

is that the relative proportion of methylated cytosine has fallen dramatically following AZA treatment, and was controlled against a well-fitting standard curve (R^2=0.9542).

Following treatment with 0.50uM RRx-001, four of eight loci were effectively demethylated; MINT, HAND, NEUROG, and THBD. Patterns of methylation at the remaining four loci remained unchanged. As was the case with demethylation with AZA, the CIMP status of DLD-1 cells treated with RRx-001 did not change, remaining HIMP-high, as hMLH and SOCS were again resistant to demethylation. Although essentially a binary test, subjectively the demethylated bands observed for HAND, NEUROG, and THBD were weaker than they were for AZA, suggesting a less efficient demethylation at these loci at an equal dose. Note that the methodology employed is not quantitative, and thus this is a qualitative observation.

This finding of potentially less efficient demethylation fits with the findings of the ELISA, where although RRx-001 did produce a demonstrable global decline in the proportion of 5-mC, it was not such a precipitous drop as that observed with AZA. At 0.50uM RRx-001 demethylation was only approximately half that of observed with the same dose of AZA, and equivalence was only reached at a dose of 0.75uM RRx-001 (just above the cytotoxic threshold). This is consistent with a previous *in vitro* study where RRx-001 was found to modulate DNMT1 and DNMT3a activity by increasing free oxygen and nitrogen radicals, thus reducing the abundance of methylation substrates in a dose dependent but dynamic manner[454]. This is opposed to the permanent sequestration of DNMT1 and thus failure of de-novo methylation during cell division brought about by AZA.

The observation that high doses of either compound cause a paradoxical rebound in global methylation must disregarded as an anomaly, primarily because these doses (>1.0uM AZA and RRx-001) are well above the cytotoxic threshold. Additionally, because the level of methylation that might be expected if the agents are acting in a targeted manner would be so low, the efficiency and therefore results of the ELISA cannot be relied upon. Furthermore, there may also be direct chemical interference with the colorimetric assay at higher doses, particularly for free-radical generating RRx-001.

4.6 Conclusions

The results of this chapter demonstrate that DLD-1 colorectal cancer cells are CIMP-high at baseline, despite the absolute proportion of 5-mC being lower than expected based on the ELISA employed. Both AZA and RRx-001 were found to be effective demethylators of DLD-1 cells, both at locus specific CpG islands that form the CIMP classification system and at a global methylation level. AZA was a much more efficient demethylator than RRx-001 based on ELISA, but both agents effectively demethylated at least half of the loci associated with CIMP status; AZA demethylating 5/8 loci, RRx-001 4/8 loci, based on methylation-specific PCR. Despite locus specific demethylation, neither agent changed the CIMP status of DLD-1 cells as 2/3 Group 1 genes remained methylated (hMLH and SOCS).

When DLD-1 cells were exposed to the same doses of AZA and RRx-001 that resulted in effective demethylation, their ability to migrate in two-dimensions and invade in three-dimensions through and ECM-like substrate were significantly restricted. These effects occurred at doses that had previously been demonstrated to be sub-cytotoxic. We can therefore infer that demethylation results in a reduced ability of DLD-1 cells to migrate and invade in a manner akin to the EMT and MET processes of *in vivo* colorectal cancers, and that this pathological process may be driven by aberrant locus specific hypermethylation. The next chapter of this thesis will therefore investigate locus specific hypermethylation as a potential epigenetic mechanism underlying the EMT/MET of colorectal cancer.

Chapter 5

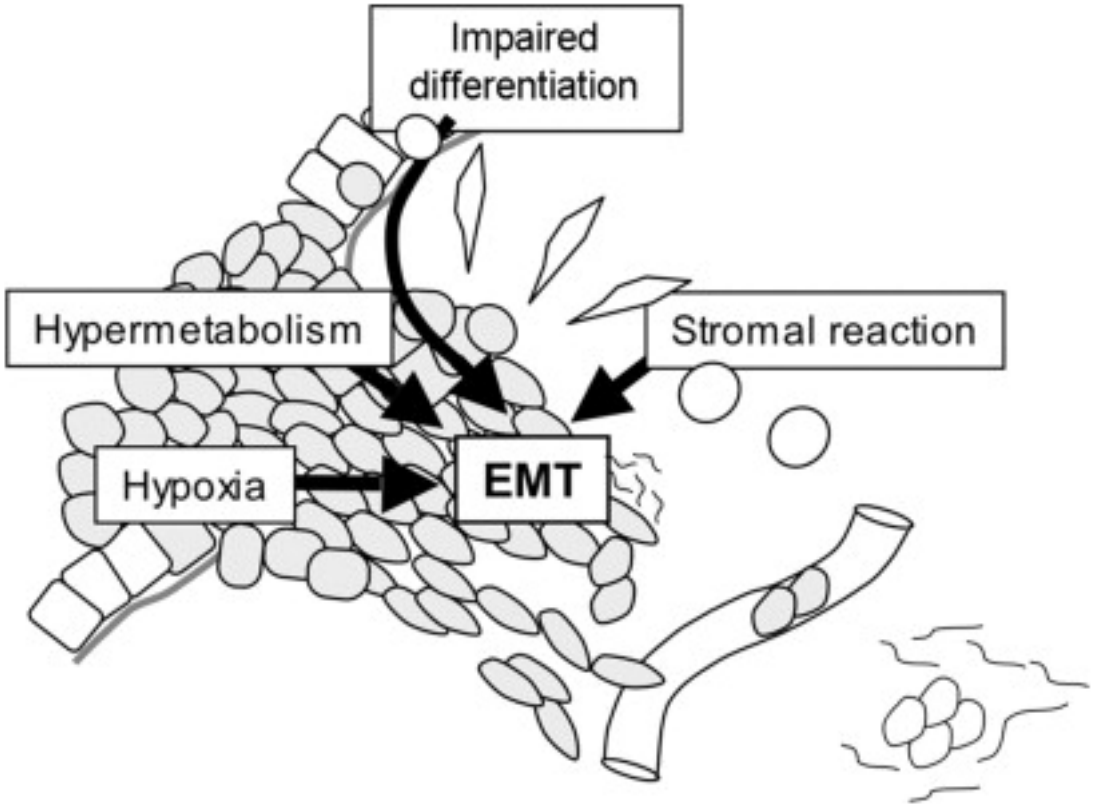
Methylation Dependent Gene Expression and the Epithelial-Mesenchymal & Mesenchymal-Epithelial Transition.

5.1 Introduction

5.1.1 The Epithelial-Mesenchymal & Mesenchymal-Epithelial Transitions

The epithelial to mesenchymal transition (EMT) is a normal physiological process whereby cells of the epithelium undergo multiple phenotypic changes to become mesenchymal-type cells; typically losing their polarity and normal cell-cell adhesions and thus allowing them to become migratory and invasive[466, 467]. During embryological development this process is key to normal tissue differentiation and organ development, and malfunction in this process can lead to abortion or congenital abnormality[407]. Cells that have undergone EMT are also more resistant to apoptosis and senescence and have a role in localised immunosuppression. EMT is also an important process in wound healing and tissue fibrosis outside of embryological development, and dysfunction may underlie pathological healing (scarring) in adult tissues[468]. The reverse process, mesenchymal to epithelial transition (MET), is also a fundamental normal physiological process that describes the migration and terminal differentiation of mesenchymal cells to polarised epithelial cells[469]. Cells undergoing EMT or MET progress through a number of stages characterised by differential expression of intracellular proteins, cytoskeletal changes, and expression of cell-surface receptors and junctional complexes (**Figure 5.1**). These phenomena are also observed in carcinoma, and thus the normal processes of EMT and MET are thought to be integral to the development of cancers[470, 471]. This is particularly important when considering the processes necessary for tumours of epithelial cell origin to first become invasive through the basement membrane, to penetrate vasculature and lymphatics, and then to seed as metastasis; as these stages of tumour progression share many similarities with the EMT and MET process[467, 472, 473].

Figure 5.1.1 The Epithelial-Mesenchymal Transition (adapted from Klymkowsky & Savagner)[474].



When considering EMT-like progression in early cancers, including CRC, it is important to recognise that the process of tumourigenesis shows plasticity from a cellular and heterogeneous from a population perspective[470]. Based on a model of early skin squamous cell carcinoma, Pastushenko and colleagues describe how induced EMT-like tumourigenesis resulted in a significant increase in the heterogeneity of expressed cell surface markers. By characterising these markers as either epithelial-associated of mesenchymal-associated the degree of EMT-progress was able to be characterised, revealing sub-populations of tumour cells displaying differing degrees of transition, described as degrees of *stemness*. This concept has been previously reported by authors when investigating the heterogeneity of early invasive tumours and the observation that some cells undergoing an EMT-like transformation are able to transit between different states[475]. Even when tumours have become well established (by size criteria), solid-type (non-diffuse) tumours, such as colorectal adenocarcinoma, display localised heterogeneity within the tumour mass; cells at the invasive front displaying phenotypic differentiation to those at the centre of the tumour mass[476]. This is also evident at a genetic and epigenetic level[477].

Due to the levels of observed heterogeneity, some authors have advocated a classification system for the degree of EMT-like phenotypes in carcinoma based on characteristic such as cell polarity, cell-cell adhesion, loss of keratin expression, and upregulation of vimentin[474]. This system is based on EMT-like phenotypic changes that are conserved across different tumour types (including colorectal adenocarcinoma, lobular breast carcinoma, melanoma, and sarcomas) and may serve as an exercise in classifying tumour from an academic perspective. However, there is currently no clinical application for this system, and no clinicopathological correlation has been attempted, although the conservation of some phenotypic features serves as a starting point for understanding some of the genetic and cellular events that underlie the process.

In healthy tissues, the adhesion between epithelial cells is predominated by E-cadherin, which is itself linked to the actin component of the cytoskeleton via its intracellular domain and associated proteins β- & α-catenin. During physiological and cancer-related EMT, loss of E-cadherin cell-cell interaction is regarded as a critical step, and E-cadherin as a "master regulator" of the loss-of-adhesion component of the EMT process[472]. Normal regulation of E-cadherin relies on a highly complex set of interactions between canonical intracellular signalling pathways, although β-catenin, RAS, and src are thought to play a central role[478]. As previously discussed in **Section 1.3.2**, activation of the APC/β-catenin pathway through mutation and pathological RAS signalling are regarded as key early steps in the classical adenoma-carcinoma pathway of CRC, and may result in loss of E-cadherin function. Of note, very few cancers are thought to originate with a primary mutation in E-cadherin itself, although some diffuse-type cancers (notably lobular breast carcinoma) have been associated with heterogeneous methylation of CpG island associated with E-cadherin gene CDH1 on the long arm of chromosome 16[479].

As well as loss of E-cadherin, EMT/invasive changes are induced in response to growth factor signalling, specifically at receptor tyrosine kinases in response to insulin-like growth factor (IGF) or transforming-growth factor β (TGF-β)[480]. Response to IGF is thought to dominate the normal EMT process by activation of the RAS/MAPK, PIK3AK/AKT, and src pathways, although pathological carcinoma invasion in response to IGF is primarily mediated through a β-catenin pathway. As well as disrupting cell-cell adhesion by effects on E-cadherin, IGF-II is able to induce EMT by upregulation of β-catenin/TCF-3 target genes including cyclin-D1 and c-myc; which themselves are a cell-cycle protein important at G1 (associated with VEGF

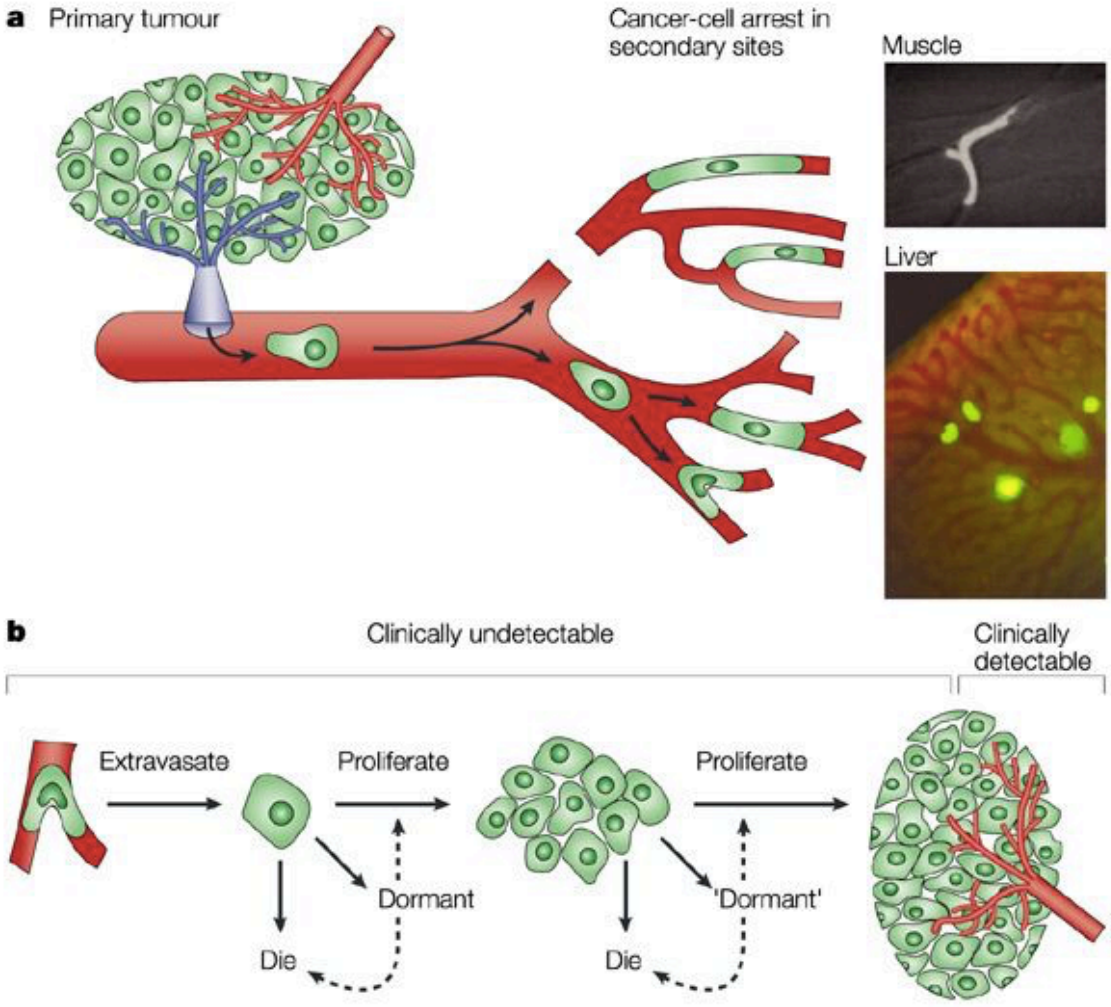
mediated angiogenesis in tumours) and a transcription factor (proto-oncogene associated with cell proliferation in cancers), respectively[481]. TGF-βs are regulatory growth factors secreted by stromal cells and are essential to healthy EMT (intrauterine global loss of TGF-βs is fatal) and contribute to normal crypt architecture in the small bowel and colon[482]. In healthy colon the TGF-β family of signalling ligands normally act on epithelial cells to suppress cell proliferation and tumour development via their effects on SMAD expression and reciprocal β-catenin suppression, and by the activation of cyclin-dependent kinase inhibitors[483]. However, in the context of early tumours undergoing and EMT-like progression, TGF-βs can act conversely to suppress the stromal anti-tumoural inflammatory response mediated by immune cells, thus facilitating invasion[484, 485].

During EMT-like invasion, cancerous cells must change their cytoskeletal structure to facilitate migration through the ECM. Although also integral to the E-cadherin regulated cell-cell adhesion, cytoskeletal components such as cytokeratins (intermediate filaments: IF) are critical to cell division & migration during EMT and are highly conserved across tissue and tumour types[486, 487]. The IFs themselves are a huge family of highly conserved proteins that are differentially expressed in different tissue types and are typically sub-divided into groups. Groups I and II are typically found in epithelial cells and the expression of specific cytokeratins in this group has aided in the characterisation of cancers, especially when identifying the source of metastasis[488]. However, group III IFs such as desmin and vimentin are more typically expressed in stromal cells such as fibroblasts but have been found to be over-expressed in a variety of epithelial-type cancers such as bladder and colorectal cancer, particularly following metastasis[489, 490]. The increased expression of stromal cytoskeletal proteins is another indication that cells undergoing malignant transformation initially progress through an EMT-like process. In colorectal cancer, EMT-like induction of cytoskeletal changes is again primarily mediated via the TGF-β pathway and is downstream effects on the SMAD3 and SMAD4 transcription factors, and the wnt/β-catenin pathway and downstream effects on SLUG and SNAIL[490]. Despite increases in expression of group III IFs in epithelial cancers, the use of proteins such as vimentin as biomarkers in colorectal cancer had not been adopted, largely due to poor sensitivity during early disease stages and lack of superiority in stage IV disease over traditional markers such as CEA[491, 492].

As well as loss of normal cell-cell adhesion and changes to the cytoskeleton, cells undergoing physiological and pathological EMT demonstrate loss of normal cell polarity. Cell polarity is normally maintained by polarity regulator complexes (PRCs) such as *Bazooka*, *Crumbs*, and *Scribble*, which are not only highly conserved across cell types but also between species[493]. As well as maintaining basal-apical polarity, these complexes also have an important role in cell migration and proliferation, both in EMT and in carcinogenesis. Under normal physiological conditions, polarity is maintained by the interaction of PRCs with components of the basement membrane and stroma such as laminin, collagens I-IV, and other integrins. Changes to the cellular microenvironment can, however, induce EMT in a number of epithelial call types in a process mirrored by early cancer transformation[494]. This is demonstrated by the observation that blockade of normal β1 integrin binding stimulates expression of the malignant phenotype in some pre-malignant epithelial cancers[495]. In colorectal cancer, PRC apical protein kinase C (aPKC) may be found to be over-expressed as a result of RAS mutation, and has been found to be a critical factor in regulating EMT[496, 497]. The relationship between aPKC over-expression and dysfunction of PRCs such as *Scribble* seem to be key to this element of early cancer development, and may act via a common pathway of ErbB2 transcription and subsequent HER2 expression, leading to downstream effects on MAPK and PIK3AK signal transduction pathways[498]. The central role of HER2 in CRC may be reflected in HER2 expression being a factor in predicting loss of treatment response to anti-EGFR therapies, and may itself be a future therapeutic target[499].

The reverse process of MET is less well characterised than EMT, and the genetic and cellular events that underlie the observed phenotypic changes are not well understood. It should also be noted that when considering healthy physiological MET the original mesenchymal cells become terminally differentiated epithelial cells, whereas the cells of a tumour metastasis remain abnormally differentiated[500]. There is, however, an acceptance that tumour cells that have successfully metastasised have adhered to and penetrated the host tissue vascular or lymphatic wall, migrated to the parenchyma, and seeded in their metastatic niche, after which they must proliferate to form a metastasis. The limiting factor in this process is deemed to be the ability of the micrometastasis to induce angiogenesis (**Figure 5.2**)[501]. This phenotypic change may occur after a long period of tumour cell dormancy, sometimes many years after the primary tumour has been treated. The complexity of this process perhaps underlies the estimation that only 0.01% of circulating tumour cells form a secondary tumour[502].

Figure 5.1.2 The Metastatic Process (adapted from Chambers, *et.al.*)[502].



Once epithelial cells have undergone the EMT-like process of loss of normal cell-cell adhesion, loss of polarity, and changed their cytoskeletal architecture they are more able to negotiate the basement membrane and stromal layers. Fundamentally this is the process that facilitates colorectal cancers to progress form tumourous cells in situ (Tis) to more advanced tumours of the bowel wall (T1-4), to invade blood vessels and lymphatic, and eventually metastasise to local lymph nodes and more distant sites. Another requirement for tumours progress on this pathway is the ability to degrade the extracellular matrix.

5.1.2 Metalloproteinases & Cancer

The metalloproteinases (MMPs) are a large group of proteolytic enzymes capable of degrading all of the protein components of the extracellular matrix (ECM), as well as some growth factors. This large family is sub-divided into groups dependent on the preferred target substrate of each MMP, such as the collagenases (MMP1, -8, -13) or the gelatinases (MMP2, -9)[503]. Throughout the body of scientific literature, MMPs are referred to variably by their MMP assignment, their substrate assignment, or common names, leading to significant potential for confusion. One such example is collagen-IV-specific MMP2, which is variably also known as Gelatinase-A or 72kDa Type-IV collagenase. For the purpose of this thesis each MMP will be referred to by its MMP assignment (e.g. MMP2, MMP9, etc). MMPs are summarised in **Figure 5.3**. Most MMPs are released into the ECM, often in the form of an inactive pro-enzyme (zymogen), after which they are activated and free to act upon the surrounding substrate. However, some sub-groups of MMP can be found attached to the cell surface (the GPI-anchored MMPs) or as transmembrane proteins (the transmembrane-type MMPs)[504].

Figure 5.1.3 Matrix Metalloproteinases (MMPs) (adapted from Stamenkovic)[505].

MMP#	Common name	Mr (kDa)	Substrates	Cell surface	Docking mechanism
1	interstitial collagenase, collagenase-1	52/43	collagen I, II, III, VII, X, IGFBP	yes	EMMPRIN (CD147)
2	gelatinase A	72/62	gelatin, collagen I, IV V, X, laminin, IGFBP latent TGF-β	yes	MT1-MMP/TIMP-2 $\alpha v\beta 3$ integrin
3	stromelysin-1	52/43	collagen III, IV, V, IX X, gelatin, laminin proteoglycans, fibronectin, elastin, E-cadherin, IGFBP, HB-EGF, perlecan, proMMP-13	not shown	
7	matrilysin	28/19	gelatin, fibronectin, laminin, collagen IV, proteoglycans FasL, HB-EGF, pro-MMP-1	yes	heparan sulfate proteo-glycans, CD44v3
8	neutrophil collagenase, collagenase-2	51/42	collagen I, II, III, VII, X	not shown	
9	gelatinase B	92/83	gelatin, collagen I, IV,V, X, IGFBP, latent TGF-β	yes	CD44, collagen IV, others?
10	stromelysin-2	52/43	collagen III, IV, IX, X gelatin, proteoglycans laminin, pro-MMP-1 pro-MMP-13	not shown	
11	stromelysin-3	51/44	α-1-antiprotease, IGFBP	not shown	
12	metalloelastase	52/43	elastin, pro-MMP-13	not shown	
13	collagenase-3	52/42	collagen I, II, III,IV,VII, X, XIV, tenascin, aggrecan, fibronectin, pro-MMP-9	not shown	
14	MT1-MMP	64/54	gelatin, collagen I, fibrin, fibronectin, proteoglycans, laminin, pro-MMP-2. Pro-MMP-13	yes	transmembrane domain
15	MT2-MMP	71/61	fibronectin, laminin, tenascin, pro-MMP-2 pro-MMP-13	yes	transmembrane domain
16	MT3-MMP	66/56	collagen III, gelatin fibronectin, pro-MMP-2	yes	transmembrane domain
17	MT4-MMP	54	unknown	yes	transmembrane domain
18/19	RASI-1	55/47	unknown	not shown	
20	Enamelysin	52/43	amelogenin	not shown	

The role of MMPs is to modulate the structural components of the ECM; predominantly, collagens and gelatins. This is an important part of healthy embryological development and organogenesis, as well as tissue homeostasis, remodelling, and repair in mature tissues[506]. This is particularly pertinent when considering wound healing and the migration and invasion of mesenchymal cells through he ECM, although most cells in their base state do not express MMPs strongly. Transcriptomal control of MMPs is partially mediated by growth factors, hormones, cytokines, and cellular transformation, including soluble suppressive factors such as TGF-β, retinoic acid, and glucocorticoids. However, the primary induction of MMP expression is mediated by integrins or cell-cell interactions; particularly cells of the immune system[507, 508]. The extracellular action of MMPs further regulated by the balance of inhibitory factors; specifically, α-macroglobulins and MMP-specific tissue-inhibitors of metalloproteinases (TIMPs)[509].

In addition to their roles is healthy tissue maintenance, MMPs also have a long-established role in carcinogenesis[505]. In relation to epithelial-derived tumours, such as CRC, the first stage of tumour invasion is through the basement membrane, which itself is primarily composed of type-IV collagen, and thus much interest has been directed towards MMP2 and MMP9[510]. The role of MMP2 has been investigated specifically in relation to colorectal cancer, where increased expression has previously been associated with worse Duke's stage[511]. Similarly, TIMP2, the specific inhibitor of MMP2, has been associated with increased likelihood of localised disease spread in colorectal cancer, although MMP2 and MMP9 were not found to be significant[512]. At best, the role and significance of increased MMP2 and MMP9 expression in solid cancers, or their respective inhibitors, is not consistent. Several tumour types have shown association between worsening stage and/or tumour biology associated with increased MMP2 expression, although MMP2 has failed to be reliable as a diagnostic tumour marker or in stratifying clinical outcomes[513].

A more recent perspective on the role of collagen-IV specific MMPs in colorectal cancer has examined the failures to translate laboratory models of CRC and MMP activity to observations made *in vivo*[514]. Mook and colleagues consider that MMPs and early invasive CRC are interacting with a complex stromal environment *in vivo*, and that the previous modelling of CRC (specifically in the application of MMP-blocking agents as potential therapy) has been inadequate in accounting for the complex cell-cell and cell-stromal interactions that partially determine MMP activity. One hypothesis suggested is that MMP inhibition may actually

contribute to carcinogenesis by preventing MMPs degrading angiogenic growth factors and cytokines. Furthermore, selective inhibition of MMP2 and MMP9 may also contribute to carcinogenesis in CRC by preventing macrophage and other inflammatory cell migration to early tumours, thus reducing anti-tumoural response. The failure of MMP inhibitors as anti-cancer therapies, and a growing appreciating that these failures may be due to a poor appreciation of the role of these proteinases in the immune response, have subsequently prompted investigators to examine whether MMP inhibitors may have a role in inflammatory diseases such as rheumatoid arthritis[515].

Surprisingly, there has been little investigation of methylation of MMPs and TIMPs relating to any type of cancer, and almost none relating to colorectal cancer. A single study identified relative hypermethylation and subsequent silencing of MMP16 (a transmembrane MMP) in *in vitro* colorectal cancers and in colorectal cancer cell lines (SW480, DLD-1, and LoVo)[516]. Treatment with AZA restored expression of MMP16 following demonstrable locus-specific demethylation. Further studies have highlighted a potential role for epigenetic mechanisms (including methylation and chromatic restructuring) in the regulation if MMPs in other diseases, including ischaemic stroke, diabetic retinopathy, and rheumatoid arthritis[517-519].

5.1.3 Differential Gene Expression

Phenotypic changes in epithelial cells undergoing EMT-like progression to early invasive cancers will change the expression of genes required to effect this transformation. As this process defines a malignant transformation, the genetic factors that bring about the change in gene expression can thus be assumed to carcinogenic; whether chromosomal, mutational, or epigenetic. However, due to the high-level of interlinkage between canonical intracellular signalling pathways, care must be taken when interpreting expressional changes associated with signalling, and thus end-effect changes in expression (such as those that define EMT or the relationship to the ECM) may provide better indicators of invasive transformation[520].

One method of examining differential gene expression is to examine messenger RNA (mRNA) profiles; relative increases or decreases in specific gene mRNA provide an insight into biologic mechanisms, and may be attributable to specific genetic events, exposure to genotoxic agents, or therapies. This methodology is widely applied in toxicogenomics, where cells are exposed to putative genotoxins and expressional changes associated with exposure can therefore indicate a mechanism of carcinogenic action[521]. The process may also be applied in reverse, where exposure to a potentially therapeutic agent may result in differential gene expression that may be regarded as effecting an anti-tumoural phenotypic change or cell death. The combination of these molecular techniques with chemo- and bioinformatics can also reveal expressional changes associated with therapeutic agents that are not evident at a phenotypic level[522, 523].

When screening for expressional changes, a high-throughput wide-net approach is usually employed in the first instance to capture a potentially relevant expressional event, which can then be focused-down upon by more targeted methods. One such method of initial screening is through microarrays[524]. Microarrays exploit the relative binding saturation of fluorescently labelled target RNA to specific pre-loaded oligonucleotide primers on rigid surfaces, causing differential fluorescence to indicate relative expression. In this format microarrays are commonly known as gene chips and have the ability to screen multiple genes (from the tens to the many hundreds, depending on the platform). Once differential expression has been examined, clustering techniques (supervised or unsupervised data analysis) may be used to identify genes with a similar response to stimulus, or which genes best indicate the application of specific stimuli[525].

This process has been refined further to incorporate quantitative real-time PCR (qRT-PCR) techniques to the basic array format and is now considered the gold-standard in quantitative assay screening for expressional change[526, 527]. This process relies upon real-time observation of relative fluorescence during exponential amplification of complimentary cDNA, and thus both an internal and external control for expression must be employed, as well as a pre-amplification stage of cDNA synthesis from mRNA. Despite the additional complexity, qRT-PCR has been shown to be an accurate and precise method of assessing gene expression[528]. If a biologic process is suspected, pathway-specific microarrays based upon genes known to be important to that processes may facilitate in the initial screening by narrowing the target spectrum of genes, increasing efficiency.

qRT-PCR can also be used to validate the array data once it has suggested specific target genes of interest and may also demonstrate dose-dependent expressional change when examining the effects of genotoxic of therapeutic agents. The selection of target genes for qRT-PCR is based upon array data, but also consideration of the biologic process being investigated and the complexity of cellular signalling; thus, differential extracellular enzyme expression may provide more illuminating to phenotypic changes than, for instance, a canonical signalling protein with multiple downstream effects. The expressional changes that may be observed in array and specific qRT-PCR may be confirmed further by standard quantitative protein techniques, such as Western blotting.

For the purposes of this study, the Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis was chosen as the PCR array[527]. There were several factors influencing the choice of this array, especially considering there are EMT-specific arrays provided by the same manufacturer that might have provided a more specific insight into the expressional events that underlie EMT-like transformation of an early cancer. However, the overarching narrative of this thesis is to examine specifically the biological processes that underlie extramural vascular invasion in rectal cancer, particularly in the context of locally advanced disease. These processes depend not just on and EMT-like process enabling invasion of the submucosa, but also invasion of other tissues, including blood vessels and lymph nodes. A decision was made, therefore, to look more closely at the extracellular remodelling, locally invasive, and locally metastatic abilities of DLD-1 cells (derived from a Dukes C adenocarcinoma), as opposed to early EMT-like changes such as loss of cell polarity and cell-cell adhesion. Clearly there is a high degree of overlap between EMT and local invasion from the point of view of underlying

biologic process, and thus some of the same genes are examined on the corresponding EMT and metastatic arrays. However, on balance, the coverage of likely genes of interest was more appropriate on the human tumour metastasis array than the EMT array. This was particularly the case for MMPs and TIMPs, with only MMP2, -3, & -9, and TIMP1 being examined on the EMT array, whilst MMP7, -10, -11, & -13, and TIMPs -2, -3, & -4 are all examined on the metastasis array in addition to those examined on the EMT array. Additionally, the APC, KRAS, and SMAD genes are also examined on the metastasis array but are absent on the EMT panel. Cadherins and Type IV collagen are present on both. A full list of gene targets on the Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis focused array is presented in **Figure 5.1.4**.

Figure 5.1.4 Genes on the Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis.

Position	UniGene	GenBank	Symbol	Description
A01	Hs.158932	NM_000038	APC	Adenomatous polyposis coli
A02	Hs.100426	NM_015399	BRMS1	Breast cancer metastasis suppressor 1
A03	Hs.251526	NM_006273	CCL7	Chemokine (C-C motif) ligand 7
A04	Hs.502328	NM_000610	CD44	CD44 molecule (Indian blood group)
A05	Hs.527778	NM_002231	CD82	CD82 molecule
A06	Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
A07	Hs.116471	NM_001797	CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)
A08	Hs.171054	NM_004932	CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)
A09	Hs.512599	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
A10	Hs.162233	NM_001273	CHD4	Chromodomain helicase DNA binding protein 4
A11	Hs.508716	NM_001846	COL4A2	Collagen, type IV, alpha 2
A12	Hs.143212	NM_003650	CST7	Cystatin F (leukocystatin)
B01	Hs.208597	NM_001328	CTBP1	C-terminal binding protein 1
B02	Hs.534797	NM_001903	CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa
B03	Hs.632466	NM_000396	CTSK	Cathepsin K
B04	Hs.716407	NM_001912	CTSL1	Cathepsin L1
B05	Hs.522891	NM_000609	CXCL12	Chemokine (C-X-C motif) ligand 12
B06	Hs.846	NM_001557	CXCR2	Chemokine (C-X-C motif) receptor 2
B07	Hs.593413	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4
B08	Hs.22393	NM_003677	DENR	Density-regulated protein
B09	Hs.523329	NM_004442	EPHB2	EPH receptor B2
B10	Hs.434059	NM_001986	ETV4	Ets variant 4
B11	Hs.374477	NM_005243	EWSR1	Ewing sarcoma breakpoint region 1
B12	Hs.481371	NM_005245	FAT1	FAT tumor suppressor homolog 1 (Drosophila)
C01	Hs.165950	NM_002011	FGFR4	Fibroblast growth factor receptor 4
C02	Hs.646917	NM_002020	FLT4	Fms-related tyrosine kinase 4
C03	Hs.203717	NM_002026	FN1	Fibronectin 1
C04	Hs.333418	NM_014164	FXYD5	FXYD domain containing ion transport regulator 5
C05	Hs.82963	NM_000825	GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)
C06	Hs.396530	NM_000601	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)
C07	Hs.44227	NM_006665	HPSE	Heparanase
C08	Hs.37003	NM_005343	HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
C09	Hs.90753	NM_006410	HTATIP2	HIV-1 Tat interactive protein 2, 30kDa
C10	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)
C11	Hs.83077	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)
C12	Hs.126256	NM_000576	IL1B	Interleukin 1, beta
D01	Hs.524484	NM_002206	ITGA7	Integrin, alpha 7
D02	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
D03	Hs.95008	NM_002256	KISS1	KiSS-1 metastasis-suppressor
D04	Hs.208229	NM_032551	KISS1R	KISS1 receptor
D05	Hs.505033	NM_004985	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
D06	Hs.599039	NM_006500	MCAM	Melanoma cell adhesion molecule
D07	Hs.484551	NM_002392	MDM2	Mdm2 p53 binding protein homolog (mouse)
D08	Hs.132966	NM_000245	MET	Met proto-oncogene (hepatocyte growth factor receptor)
D09	Hs.444986	NM_006838	METAP2	Methionyl aminopeptidase 2

Position	UniGene	GenBank	Symbol	Description
D10	Hs.651869	NM_002410	MGAT5	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase
D11	Hs.2258	NM_002425	MMP10	Matrix metallopeptidase 10 (stromelysin 2)
D12	Hs.143751	NM_005940	MMP11	Matrix metallopeptidase 11 (stromelysin 3)
E01	Hs.2936	NM_002427	MMP13	Matrix metallopeptidase 13 (collagenase 3)
E02	Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
E03	Hs.375129	NM_002422	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
E04	Hs.2256	NM_002423	MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)
E05	Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
E06	Hs.525629	NM_004689	MTA1	Metastasis associated 1
E07	Hs.700429	NM_014751	MTSS1	Metastasis suppressor 1
E08	Hs.202453	NM_002467	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
E09	Hs.437922	NM_005376	MYCL1	V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)
E10	Hs.187898	NM_000268	NF2	Neurofibromin 2 (merlin)
E11	Hs.118638	NM_000269	NME1	Non-metastatic cells 1, protein (NM23A) expressed in
E12	Hs.9235	NM_005009	NME4	Non-metastatic cells 4, protein expressed in
F01	Hs.279522	NM_006981	NR4A3	Nuclear receptor subfamily 4, group A, member 3
F02	Hs.466871	NM_002659	PLAUR	Plasminogen activator, urokinase receptor
F03	Hs.409965	NM_002687	PNN	Pinin, desmosome associated protein
F04	Hs.500466	NM_000314	PTEN	Phosphatase and tensin homolog
F05	Hs.408528	NM_000321	RB1	Retinoblastoma 1
F06	Hs.494178	NM_006914	RORB	RAR-related orphan receptor B
F07	Hs.449909	NM_002295	RPSA	Ribosomal protein SA
F08	Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
F09	Hs.436687	NM_003011	SET	SET nuclear oncogene
F10	Hs.12253	NM_005901	SMAD2	SMAD family member 2
F11	Hs.75862	NM_005359	SMAD4	SMAD family member 4
F12	Hs.195659	NM_005417	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
G01	Hs.514451	NM_001050	SSTR2	Somatostatin receptor 2
G02	Hs.371720	NM_003177	SYK	Spleen tyrosine kinase
G03	Hs.475018	NM_005650	TCF20	Transcription factor 20 (AR1)
G04	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1
G05	Hs.633514	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2
G06	Hs.644633	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3
G07	Hs.591665	NM_003256	TIMP4	TIMP metallopeptidase inhibitor 4
G08	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
G09	Hs.654481	NM_000546	TP53	Tumor protein p53
G10	Hs.155942	NM_002420	TRPM1	Transient receptor potential cation channel, subfamily M, member 1
G11	Hs.160411	NM_000369	TSHR	Thyroid stimulating hormone receptor
G12	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A
H01	Hs.520640	NM_001101	ACTB	Actin, beta
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

5.2 Aims & Objectives

This chapter will examine the gene-expressional effects of demethylation of colorectal cell lines *in vitro* by AZA and RRx-001. Firstly, a gene qRT-PCR array will be employed to identify genes of interest, with a focus on those genes important in the EMT and locally invasive processes. Secondly, a more in-depth investigation of differential expression of genes identified by the array will be performed to confirm array results, and to examine for any exposure-expression relationship between identified genes and AZA & RRx-001.

5.3 Methods

DLD-1 cells were cultured in control conditions or exposed to AZA and RRx-001 according to the protocols described in **Sections 2.3** and **4.3.1.2**. Treatment arm doses of AZA and RRx-001 were 0.50uM. For the purposes of the array, only AZA at 0.50uM for 72hrs exposure was used against control, as this provided a baseline for investigation potential differential gene expression in a well characterised compound with a specific mode of demethylation. For subsequent gene-specific qRT-PCR, RRx-001 and an additional dose-exposure of 24hrs AZA were employed. The purpose of this was to examine for any temporal effect of demethylating agent AZA, as it relies on cell proliferation to effect demethylation. This was the only modification.

5.3.1 RT^2 Profiler PRC Array Gene Expression

5.3.1.1 RNA Extraction & Purification

RNA was extracted from DLD-1 cells by employing the Qiagen RNeasy RNA kit. There was no deviation from the product protocol. The maximum RNA haves from this system is 100µg per sample, and thus a maximum of $5x10^6$ cells (as recommended in product literature) were used in each sample to prevent over-saturation of the system. DLD-1 cells were first trypsinised and washed twice with PBS before undergoing RNA extraction. All reagents were prepared as per the kit protocol and RNA extraction was performed under clean laminar-flow conditions using standard precautions to prevent cross-contamination / fouling of reaction.

Firstly, 600uL of Buffer RLT was added to cell pellets in a microcentrifuge eppendorf containing pelleted cells and vortexed thoroughly until no cell clumps were visible and until fully homogenized (approximately 3 minutes). Following homogenization, 600uL of 70% ethanol was then added and the suspension mixed thoroughly by pipetting. Of the resulting suspension, 700uL of was then transferred to spin columns placed in a 2ml collection tube and centrifuged at 10,000rpm for 15 seconds. The flow-through was discarded. 700uL of Buffer RW1 was added to the spin column (replaced in the emptied collection tube) and was centrifuged again at 10,000rpm for 15 seconds. The flow-through was discarded. 500uL of Buffer RPE was then added to the spin column and centrifuged at 10,000rpm for 15 seconds. The flow-through was discarded. A further 500uL of Buffer RPE was added to the spin column but this time centrifugation at 10,000rpm was for 2 minutes to ensure drying of the RNA collection membrane and full elimination of any residual ethanol. Spin columns were then transferred to a fresh 1.5ml collection tube and 50uL of nuclease-free water (DEPC-free) was

added directly to the spin column membrane. Spin columns were centrifuged at 10,000rpm for 1 minute to elute the RNA. Harvested RNA was then quantified and its purity checked by spectrophotometery (Nanodrop) at 260 nm. RNA was stored at -20^{o}C.

5.3.1.2 cDNA Synthesis

The Qiagen RT^2 First Strand Kit was used to generate cDNA from mRNA as it is the validated kit for use in conjunction with the RT^2 Profiler Array. There was no deviation from the published product protocol. 0.5ug of total RNA was used for each sample as recommended by the product literature, and thus input of total RNA was standardised based on the quantified RNA harvest as specified above. Reagents were prepared as specified in the protocol.

The gDNA elimination cocktail was made by the combination of 5ug RNA with 2uL of Buffer GE and a variable amount of nuclease-free water in a clean eppendorf to make a total volume of 10uL. The mixture was mixed by pipetting and then incubated for 5 minutes at 42^{o}C, and then transferred to ice for 1 minute. 10uL of reverse-transcription cocktail was then added to each gDNA elimination cocktail and mixed by pipetting. This mix was then incubated at 42^{o}C for 15 minutes to facilitate cDNA synthesis and then the reaction stopped by incubation at 95^{o}C for 5 minutes. 91uL nuclease-free water was then added to each sample, mixed, and the sample kept on ice before proceeding directly to the RT^2 protocol.

5.3.1.3 RT^2 Profiler Array

The Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis (cat. no. 330231 PAHS-028ZA) was employed for expressional analysis. There was no deviation from the published product protocol. Reagents were prepared as specified in the protocol. RT^2 SYBR Green Mastermix (containing HotStart DNA Taq Polymerase) was employed to ensure accurate results. As the chosen array is a 96-well format plate all volumes were calculated correspondingly.

The PCR components were mixed to a total volume of 2700uL in a 5ml tube accordingly; 2x RT^2 SYBR Green Mastermix 1350uL, cDNA product 102uL, nuclease-free water 1248uL. 25uL of the PCR components was then added to each well of the profiler array using an 8-channel pipettor, ensuring fresh tips for each well to avoid cross-contamination of well contents. The array wells were then sealed with the supplies Optical Thin-Wall 8-cap strips. The plate was then centrifuged for 1 minute at 12,000rpm and the plate examined to ensure no

bubbles were present at the base of the wells, and then placed on ice while the RT-PCR cycler was programmed. As a Bio-Rad (USA) IQ-5™ Real-Time PCR System was employed, cycling conditions were set as shown in **Table 5.3**. **Figure 5.3** demonstrates the plate layout for the Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis.

Table 5.3 Cycling conditions for qRT-PCR when employing the RT^2 Profiler Array.

Cycles	Duration	Temperature	Comment
1	10 minutes	95°C	HotStart DNA Taq Polymerase activation
40	15 seconds 1 minute	95°C 60°C	Exponential amplification & fluorescence data collection

Figure 5.3 Plate layout for Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis. Please refer to **Figure 5.1.4** for corresponding gene information.

	1	2	3	4	5	6	7	8	9	10	11	12
A	APC	BRMS1	CCL7	CD44	CD82	CDH1	CDH11	CDH6	CDKN2A	CHD4	COL4A2	CST7
B	CTBP1	CTNNA1	CTSK	CTSL1	CXCL12	CXCR2	CXCR4	DENR	EPHB2	ETV4	EWSR1	FAT1
C	FGFR4	FLT4	FN1	FXYD5	GNRH1	HGF	HPSE	HRAS	HTATIP2	IGF1	IL18	IL1B
D	ITGA7	ITGB3	KISS1	KISS1R	KRAS	MCAM	MDM2	MET	METAP2	MGAT5	MMP10	MMP11
E	MMP13	MMP2	MMP3	MMP7	MMP9	MTA1	MTSS1	MYC	MYCL1	NF2	NME1	NME4
F	NR4A3	PLAUR	PNN	PTEN	RB1	RORB	RPSA	SERPINE1	SET	SMAD2	SMAD4	SRC
G	SSTR2	SYK	TCF20	TGFB1	TIMP2	TIMP3	TIMP4	TNFSF10	TP53	TRPM1	TSHR	VEGFA
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

5.3.1.4 Data Analysis

Baseline was set automatically by the cycler software from cycle 2 through 2 cycles. Threshold C_T was set manually based upon the log view of the amplification plots, specifically on the lower portion of the linear amplification phase. Raw data was exported to a Microsoft Excel spreadsheet for use with SABioscinces PRC Array Data Analysis Template. A melting (dissociation) curve analysis was performed to ensure single product amplification for each well.

5.3.2 qRT-PCR of Metalloproteinases

Metalloproteinase targets were chosen based upon the results of the Qiagen RT^2 Profiler PCR Array for Human Tumour Metastasis; for full results see **Section 5.4.1**. Four sets of qRT-PCR were conducted for each gene target: Control; 24hrs 0.50uM AZA; 72hrs 0.50uM AZA; 72hrs 0.50uM RRx-001. Each culture condition was performed in three biological replicates, each of which were subject to parallel technical triplicate during PCR. mRNA was extracted, purified, and quantified by the same methods described in **Section 5.3.1.1**. A real-time one-step RT-PCR methodology was applied employing the QuantiFast SYBR Green RT-PCR kit supplied by Qiagen, eliminating a separate cDNA synthesis stage.

Six pairs of cDNA primers were designed for qRT-PCR targeting MMPs and TIMPs: MMP2, MMP9, MMP11, MMP13, TIMP2, & TIMP4. Primer design employed a dual technique of target sequence verification via the NCBI GenBank open source reference (https://www.ncbi.nlm.nih.gov/genbank/) and then oligo analysis via Beacon Designer, PREMIER Biosoft International (http://www.premierbiosoft.com/qpcr/index.html). Oligos were also cross-referenced using Primer-BLAST open access software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)[529]. β-actin was used as a housekeeping gene in order to provide reference for quantitative analysis (sequence supplied and previously validated in-house). Primer sequences are listed in **Section 2.2.5**. No primer demonstrated dimerization, hairpins, or other significant erroneous pairing events, and each had an efficiency ≥90% and a melting temperature T_m ≥70°C.

qRT-PCR reactions were set-up individually in a 96-well plate with the following components: 2x QuantiFast SYBR Green Mastermix 12.5uL, Primer A 0.5uL, Primer B 0.5uL, QuantiFast RT Mix 0.25uL, template RNA variable volume to control for 100ng/reaction, nuclease-free water variable volume to make-up total reaction volume to 25uL. In addition to the experimental wells performed in triplicate, a standard curve was constructed by serial dilution (concentrations 1.0, 0.1, 0.001, 0.0001) for each primer and each plate. Reagents were kept on ice throughout to prevent degradation of mRNA. Plates were sealed with biofilm. The plate was then centrifuged for 1 minute at 12,000rpm and the plate examined to ensure no bubbles were present at the base of the wells, and then placed on ice while the RT-PCR cycler was programmed. As a Bio-Rad (USA) IQ-5™ Real-Time PCR System was employed, cycling conditions were set as shown in **Table 5.4**.

Table 5.4 Cycling conditions for qRT-PCR when employing the QuantiFast SYBR Green RT-PCR kit and gene-specific cDNA primers.

Cycles	Duration	Temperature	Comment
1	10 minutes	50°C	Reverse transcription
1	5 minutes	95°C	HotStart DNA Taq Polymerase activation
35	10 seconds	95°C	Denaturation
35	30 seconds	60°C	Exponential amplification & fluorescence data collection

Baseline detection was set automatically by the cycler software from cycle 2 through 2 cycles. A standard curve was calculated based on β-actin & target gene serial dilutions. Threshold C_T was set manually based upon the log view of the amplification plots, specifically on the lower portion of the linear amplification phase. C_T values for triplicates not within 1 cycle of each other were removed, provided that there were at least 2 remaining. Raw data was exported to a Microsoft Excel spreadsheet for use with Biorad IQ5 software. A melting (dissociation) curve analysis was performed to ensure single product amplification for each well and anomalies excluded. The ΔΔCT method was used for relative quantification of PCR products[530-532].

5.4 Results

5.4.1 RT^2 Profiler PRC Array Gene Expression

No pre-amplification phase was performed. The lower limit of detection was set to C_T = 20, which was at the lower end of the Log-linear phase[533]. The average C_T (PPC) was 23.73 and 23.48 for control and test, respectively. Delta C_T (Average RTC – Average PPC) was 2.16 and 2.13 for control and test, respectively. There was insignificant genomic DNA contamination. Normalisation (from which $\Delta\Delta$ C_T will be calculated) was set automatically by the analytical software based on the housekeeping genes (HKG) beta-2-microglobulin (BM2) and ribosomal protein, large, P0 (RPLP0) (**Figure 5.4.1**). Melt-curve analysis was satisfactory for each well.

Figure 5.4.1. Geometric and average geometric mean C_T for selected housekeeping genes.

Groups	Samples	B2M	RPLP0	Geometric Mean	Average Geometric Mean
Control Group		26.61628267 4206	19.83350168 2052	22.98	22.98
Group 1		26.22507700 7783	20.04349951 6228	22.93	22.93

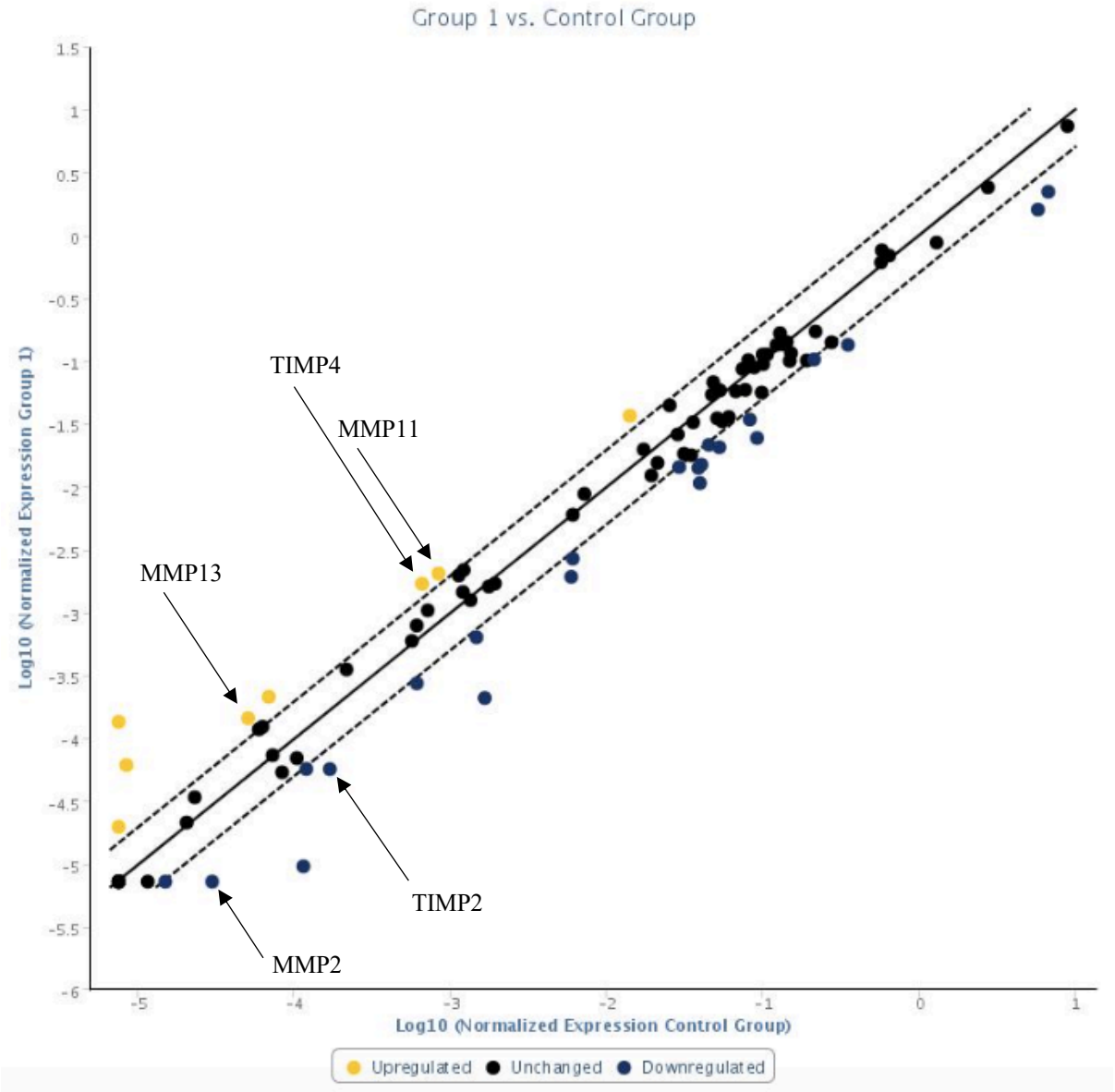
DLD-1 cells were cultured in control conditions or with AZA 0.50uM applied daily for 72hrs as described previously. At the end of the treatment period, cells were harvested and counted both by coulter counter and on a haemocytometer to ensure no unexpected toxicity had occurred, but then diluted to a maximum of 5x10^6 cells as per the RNA harvest protocol. Harvested RNA was satisfactorily quantified and its purity ensured by spectrophotometery (Nanodrop) at 260 nm. Extracted RNA was then used to assess the changes in expression of genes associated with invasion using the RT-Profiler. Spare RNA was stored at -20^{o}C for short-term availability or at -80^{o}C for archive purposes.

Figure 5.4.2 demonstrates the fold-change results for genes significantly over or under expressed compared to control, where significant fold-change threshold is set to ±2 [2^(-ΔC_T)]. Values for statistical significance (*p*)could not be calculated based on only a single repetition of the array; verification will be by targeted qRT-PCR. In order of fold-increase (relative fold-increase in brackets), the genes significantly upregulated were; CKDN2A (18.00), TRPM1 (7.28), ITGB3 (3.11), MMP13 (2.85), SSTR2 (2.63), VEGFA (2.62), TIMP4 (2.58), and MMP11 (2.45). In order of fold-decrease (relative fold-decrease in brackets), the genes significantly downregulated were; IGF1 (-11.93), FN1 (-7.91), MMP2 (-4.10), MET (-3.75), CD44 (-3.68), GAPDH (-3.57), EPHB2 (-3.07), ACTB (-3.00), FGFR4 (-2.97), PNN (-2.73), TIMP2 (-2.68), CDH1 (-2.59), NME1 (-2.54), HPRT1 (-2.40), KISS1 (-2.30), CD82 (-2.55), TNFSF10 (-2.22), CXCR2 (-2.09), TGFB1 (-2.08), FLT4 (-2.06), FXYD5 (-2.06), CST7 (-2.02). None of the remaining 66 genes (out of a total of 96 profiled genes) demonstrated significant fold-change. These results are demonstrated graphically on scatter-plot **Figure 5.4.3**.

Figure 5.4.2. Fold regulation comparison, where significant fold regulation cut-off is set to ±2.

Position	Gene Symbol	Fold Regulation	p-Value	Comments
A04	CD44	-3.68	0.000000	
A05	CD82	-2.25	0.000000	
A06	CDH1	-2.59	0.000000	
A09	CDKN2A	18.00	0.000000	
A12	CST7	-2.02	0.000000	
B06	CXCR2	-2.09	0.000000	
B09	EPHB2	-3.07	0.000000	
C01	FGFR4	-2.97	0.000000	
C02	FLT4	-2.06	0.000000	
C03	FN1	-7.91	0.000000	
C04	FXYD5	-2.06	0.000000	
C10	IGF1	-11.93	0.000000	
D02	ITGB3	3.11	0.000000	
D03	KISS1	-2.30	0.000000	
D08	MET	-3.75	0.000000	
D12	MMP11	2.45	0.000000	
E01	MMP13	2.85	0.000000	
E02	MMP2	-4.10	0.000000	
E11	NME1	-2.54	0.000000	
F03	PNN	-2.73	0.000000	
G01	SSTR2	2.63	0.000000	
G04	TGFB1	-2.08	0.000000	
G05	TIMP2	-2.68	0.000000	
G07	TIMP4	2.58	0.000000	
G08	TNFSF10	-2.22	0.000000	
G10	TRPM1	7.28	0.000000	
G12	VEGFA	2.62	0.000000	
H01	ACTB	-3.00	0.000000	
H03	GAPDH	-3.57	0.000000	
H04	HPRT1	-2.40	0.000000	

Figure 5.4.3. Scatter-plot of normalised expression for treatment (Group1) versus control, with selected individual genes highlighted.



5.4.2 qPCR of Metalloproteinases

Candidate genes were selected on the basis of the qPCR array data and conceptualisation of the EMT and EMVI process as pertinent to early invasive rectal cancer. Selected genes were MMP2, MMP9, MMP11, MMP13, TIMP2, and TIMP4. Each of these genes was considerably up- or downregulated in the array when DLD-1 cells were exposed to AZA. MMP9 was also included in this stage of the investigation as it is the partner gelatinase to MMP2. Both a 24-hour and 72-hour exposure to 0.50uM AZA was used during this stage, although only a 72-hour exposure to 0.50uM RRx-001 was employed. Quality control data for mRNA and cDNA are provided in **Appendix II**.

All results for change in expression are described in terms of relative change in expression in target gene between control and treatment arm, versus change in housekeeping gene (HKG; β-actin) between the two same arms; $\Delta\Delta C_T$. Where $\Delta\Delta C_T = 1$, there is no change in expression of target gene versus housekeeping gene, given equal exposure. $\Delta\Delta C_T < 1$ indicates relative downregulation versus HKG, $\Delta\Delta C_T > 1$ indicates relative upregulation versus HKG. $\Delta\Delta C_T$ cannot be negative, but if the target gene is not expressed at all $\Delta\Delta C_T$ will be 0 irrespective of HKG expression. Synthesised results are demonstrated in **Figure 5.4.4** and raw data provided in **Appendix III**. For each of the genes other than MMP9, no wells were excluded on the basis of C_T values in excess of 1 cycle away from each other, or on unfavourable melt curves unless specified.

5.4.2.1 MMP2

Baseline expression of MMP2 was 2.7×10^{-13} that of β-actin. Following 24hrs treatment with 0.50uM AZA, relative expression of MMP2 compared to β-actin was $\Delta\Delta C_T$ 0.50 (CI ±0.28, $p>0.05$). Following 72hrs treatment with 0.50uM AZA $\Delta\Delta C_T$ was 0.24 (CI ±0.01, $p \leq 0.001$). Following 72hrs treatment with 0.50uM RRx-001 $\Delta\Delta C_T$ was 4.79 (CI ±0.30, $p \leq 0.01$).

5.4.2.2 MMP9

qRT-PCR results for MMP9 were uninterpretable due to numerous anomalous results. Multiple wells provided C_T values in excess of 1 cycle away from their technical replicates, frequently leading to 2/3 replicates being excluded. Melt curve analysis also highlighted multiple peaks suggesting dimerization and the formation of multiple PCR products. The fact that PCR products were generated suggests that the reactions were not impeded, in which case no amplification would be expected, but that there was a problem with at least one of the primer

pair. This was confirmed by a complete re-run of the plate which again delivered multiple anomalous results.

5.4.2.3 MMP11

Baseline expression of MMP11 was $3.5x10^{-5}$ that of β-actin. Following 24hrs treatment with 0.50uM AZA, relative expression of MMP11 compared to β-actin was $\Delta\Delta C_T$ 2.25 (CI ±1.09, $p>0.05$). Following 72hrs treatment with 0.50uM AZA $\Delta\Delta C_T$ was 3.26 (CI ±1.02, $p\leq0.01$). Following 72hrs treatment with 0.50uM RRx-001 $\Delta\Delta C_T$ was 1.47 (CI ±0.32, $p>0.05$). Three wells were excluded on the basis of C_T values being in excess of 1 cycle away from each other, but this did not prevent meaningful analysis. There were no unfavourable melt curves resulting in replicate exclusion.

5.4.2.4 MMP13

Baseline expression of MMP13 was $4.0x10^{-4}$ that of β-actin. Following 24hrs treatment with 0.50uM AZA, relative expression of MMP13 compared to β-actin was $\Delta\Delta C_T$ 0.70 (CI ±0.03, $p\leq0.05$). Following 72hrs treatment with 0.50uM AZA $\Delta\Delta C_T$ was 0.77 (CI ±0.11, $p>0.05$). Following 72hrs treatment with 0.50uM RRx-001 $\Delta\Delta C_T$ was 1.32 (CI ±0.42, $p>0.05$).

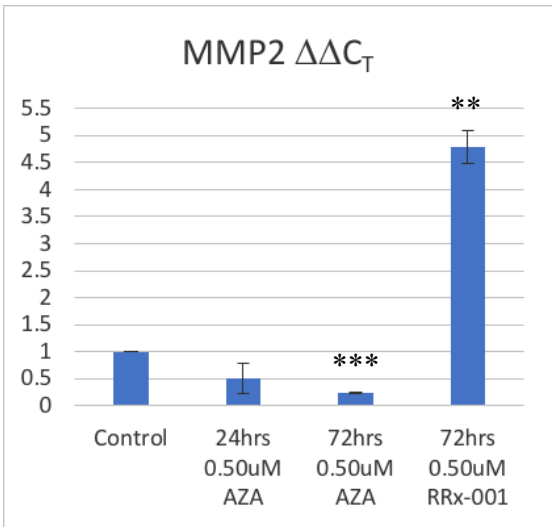
5.4.2.5 TIMP2

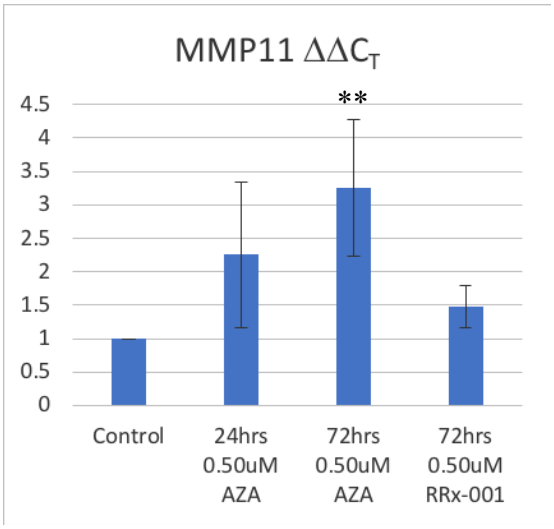
Baseline expression of TIMP2 was 0.062 that of β-actin. Following 24hrs treatment with 0.50uM AZA, relative expression of TIMP2 compared to β-actin was $\Delta\Delta C_T$ 0.87 (CI ±0.69, $p>0.05$). Following 72hrs treatment with 0.50uM AZA $\Delta\Delta C_T$ was 0.37 (CI ±0.06, $p<0.001$). Following 72hrs treatment with 0.50uM RRx-001 $\Delta\Delta C_T$ was 1.23 (CI ±0.31, $p>0.05$).

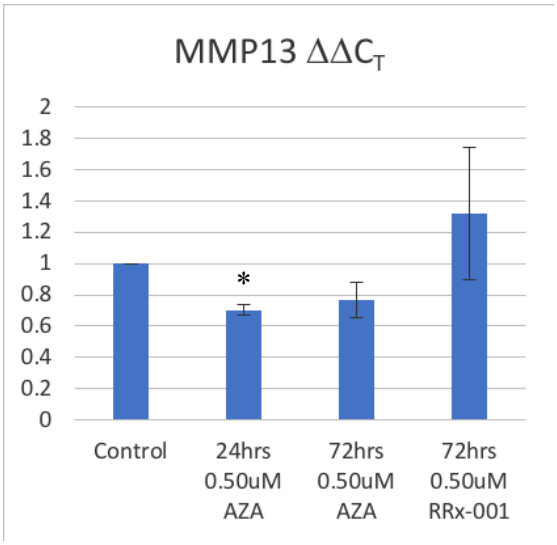
5.4.2.5 TIMP4

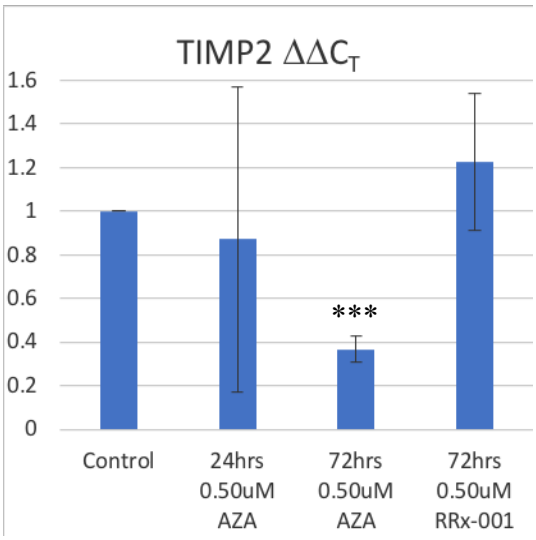
Baseline expression of TIMP4 was $1.25x10^{-7}$ that of β-actin. Following 24hrs treatment with 0.50uM AZA, relative expression of TIMP4 compared to β-actin was $\Delta\Delta C_T$ 1.45 (CI ±0.55, $p>0.05$). Following 72hrs treatment with 0.50uM AZA $\Delta\Delta C_T$ was 5.89 (CI ±1.81, $p<0.05$). Following 72hrs treatment with 0.50uM RRx-001 $\Delta\Delta C_T$ was 2.94 (CI ±0.65, $p<0.05$).

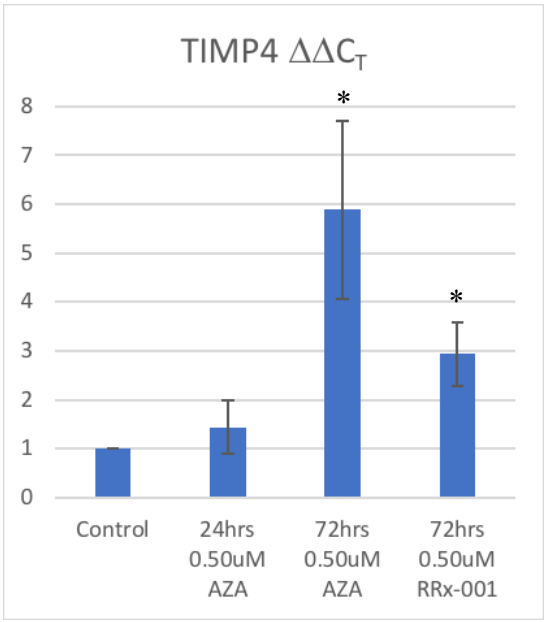
Figure 5.4.4 qRT-PCR results for $\Delta\Delta C_T$ of **a**. MMP2, **b**. MMP11, **c**. MMP13, **d**. TIMP2, and **e**. TIMP4, following treatment with AZA or RRx-001. [ΔΔCT * p≤0.05; ** p≤0.01; *** p≤0.001]











5.5 Discussion

The aim of this chapter was to investigate an association between demethylation with agents AZA and RRx-001 and any potential biologic process that may underlie the observed reduced ability of DLD-1 cells to migrate and invade following exposure to each agent. Through this insight any potential association between methylation and the specific genes that underlie the *in vivo* processes of EMT-like transformation and local invasiveness, such as those which characterises EMVI, may be gained. Results from the RT^2 Profiler PRC Array indicated that there was significant dysregulation in the MMP and TIMP genes associated with demethylation with AZA. There was also a significant decrease in cadherin expression (CDH1), but no change in the expression of APC, KRAS, SMADs. Expressional change was confirmed with qRT-PCR for each of the MMP and TIMP target genes of interest when DLD-1 cells were exposed to AZA, but the changes in expression were not consistent when cells were exposed to RRx-001. There was also a degree of temporality to the expressional effects of AZA, where a longer exposure produced a more significant change in expression in some, but not all, genes.

5.5.1 RT^2 Profiler PCR Array

The RT^2 Profiler PRC Array demonstrated that 72hrs of exposure to 0.50uM of AZA induced significant up- and downregulation (in terms of fold-change) in numerous genes associated with the EMT, locally invasive, and metastatic processes. More genes were downregulated than upregulated, but the family of genes that had undergone widespread expressional change was the metalloproteinases and their respective inhibitors. This group of enzymes are particularly important in the processes of local invasiveness as they define the ability of cells to metabolise the non-cellular components of the basement membrane and stroma[506, 534]. This is consistent with the reduced ability of DLD-1 cells to invade the ECM during invasion assays following exposure the AZA, as described in **Chapter 4**. The specific expression change of each MMP or TIMP will be discussed in greater depth in **Section 5.5.2** with corresponding qRT-PCR data. Selected other up- and downregulated genes, and those whose expression did not change, are discussed below.

5.5.1.1 Downregulated Genes

Contrary to the change in expression theoretically expected with demethylation, based upon the reduced invasive potential of DLD-1 cells after treatment with AZA, was the relative decrease (-2.59) in expression of cadherin-1 (CDH1), the gene coding for E-Cadherin. As previously discussed, E-Cadherin is a vital transmembrane protein that both regulates and effects cell-cell adhesion in epithelial cells. Mutations in CDH1 and subsequent loss of function have previously been strongly associated with epithelial cancers, particularly diffuse-type lobular breast and gastric cancers[535, 536]. Furthermore, loss of E-Cadherin and reciprocal over-expression of N-Cadherin has been associated with colorectal cancer progression[490, 537]. However, some authors have reported a complex and dynamic relationship between CDH1 expression and methylation. Graff and colleagues describe how increased methylation of CpG islands associated with CDH1 led to silencing during early invasion of the ECM (an EMT-like process) as might be expected, but that CDH1 can be re-expressed when the same cells are cultured in spheroid; suggesting a dynamic pattern of methylation and CDH1 expression.[479]. Re-expression was associated with demethylation and homotypic cell-adhesion in spheroids, and thus the authors therefore suggest that CDH1 methylation is dynamic and at-least partially dependent on stromal interactions and cell-cell relations. Decreased expression of CDH1 coupled with demethylation with AZA and decreased invasion through the ECM is therefore difficult to explain on the basis of a straightforward reciprocal relationship between methylation and expression, although the interaction with the ECM during invasion after AZA exposure may differentially alter CDH1 expression when compared to exposure during monolayer culture.

Another significantly downregulated (-11.93) gene identified on the array was Type 1 Insulin-like Growth Factor (IGF1). IGF1 has been shown to be associated with many cancer types, including colorectal cancer, and primarily exerts a tumourigenic effect via the MAPK and PIK3AK/Akt pathways[538, 539]. Activation of these pathways by IGF1 increases the risk of CRC, as well as increasing cell survival, invasion, and resistance to chemotherapy via a number of downstream mechanisms[540, 541]. Differential methylation has already been associated with colorectal cancer and irregular expression of IGF1, and also that aberrant IGF1 methylation is a shared pathway in other cancer types[542]. Downregulation of IGF1 corresponds with the observed propensity of AZA exposed DLD-1 cells to invade, as the tumourigenic effects of downstream signalling would be correspondingly downregulated.

Fibronectin (FN1) was also significantly downregulated by AZA (-7.91) based on the array data. Fibronectin is a large glycoprotein secreted into the extracellular matrix by fibroblasts and other cells, and has important roles in integrin binding, cell signalling, adhesion, migration, and proliferation[543]. Fibronectin also has an important role in wound healing, where its cross-binding with fibrin is important in clot formation and recruitment of fibroblasts[413, 544]. In colorectal cancer, over-expression of FN1 is associated with worse disease stage, and FN1-knockdown *in vitro* results in decreased expression of MMP9 and increased expression of E-Cadherin, leading to inhibition of cell migration, proliferation, and survival[545]. However, fibronectin has also been demonstrated to induce demethylation-dependent MMP2 expression in breast ductal carcinoma cells *in vitro*, and that by doing so cell migration and invasion increased[546]. No studies have yet investigated the role of methylation and fibronectin in colorectal cancer, although downregulation and corresponding reduced invasion *in vitro* would suggest it is pro-invasive as a chemoattractant, although it has been reported that fibronectin may sequester MMP2 by binding the fibronectin-like domain[547]. This hypothesis corresponds with the observation that the addition of fibronectin in the lower chamber of the Boyden chamber assay increased the Percentage Invasion through the ECM (**Chapter 4**, **Figure 4.4.7**), although the overall Invasion Index remained equal across all doses.

c-MET is a tyrosine-protein kinase coded by the MET gene that was also downregulated in the array (-3.75). MET has an important role in many cancers via activation of a number of downstream oncogenic signalling pathways including ras, PIK3A, stat, and β-catenin. MET has been associated with anti-EGFR resistance in colorectal cancer and is a poor prognostic marker for survival[548, 549]. The centrality of the c-MET in the development and progression of colorectal and other gastrointestinal cancers had led to much interest in this mechanism and the possibility for novel targeted therapies, although thus far there has been little success in translating benchtop concepts through to successful clinical trials[550]. Part of the reason for these shortcomings is the complexity of the downstream effects of MET signalling, although accurate gene-typing could provide better targeted therapies for individuals who are more likely to benefit; this is the basis for the Framework 7 MErCuRIC programme. Only a limited number of studies have investigated methylation-dependent expression of MET, although methylation events have been associated with pancreatic cancer and insulinomas, all-be-it via aberrant methylation of its regulators rather than the MET epigene itself[551].

CD44 is a cell surface glycoprotein that has important roles in cell-cell interaction and relations to the stroma, including in cell migration and adhesion. CD44 was downregulated on the assay (-3.68) following exposure to AZA. CD44 is implicated in the progression and metastasis of cancers by facilitating pro-invasive mechanisms associated with tumour cell migration, angiogenesis, and activation of growth-factor receptors[552]. CD44 has previously been shown to interact with MMPs in cancer (MMP9 and transmembrane-MMP1) to facilitate the progression of prostate cancer, and knockdown of MMP9 has resulted in a change to the CD44 isoform and the failure of cancer cells to produce invadopodia[553-555]. In colorectal cancer, CD44 is upregulated and occurs early in the adenoma–carcinoma sequence; facilitating an EMT-like progression by associated loss of APC/β-catenin tumour suppression, as well as by promoting growth-factor binging and inhibition of apoptosis[556]. None of these effects have thus far been associated with aberrant methylation events, although there may be an oblique relationship (via canonical signalling) in gastric cancer[557]. Downregulation of CD44 corresponds with the hypothesis that a degree of reversal in the loss of APC/β-catenin tumour suppression may reduce the propensity of colorectal cancer cells to invade *in vitro*.

5.5.1.1 Upregulated Genes

The most prominently upregulated gene on the array was tumour-suppressor CDKN2A (+18.0). This well characterised gene is important in cell cycle regulation and exerts anti-tumoural effects by initiating cell-cycle arrest at G2 phase (leading to apoptosis) or G1 into S phase (cell-cycle arrest) via its two coded proteins P16 and P14ARF, respectively[558]. Hypermethylation of CpG islands associated with CDKN2A has been shown to be associated with the progression of colorectal cancer and in poor prognosis, as well as in the pathogenesis of other cancer types (e.g. melanoma & pancreatic cancers)[559, 560]. However, in MSI-high colorectal tumours, methylation of CDKN2A has been demonstrated to occur in association with methylation of hMLH1 in a sub-population of tumours identified as having better overall survival, thus hypermethylation events associated with P16 function are not universally indicators of worse prognosis[561]. It must be noted, however, that this study included a limited number of patients (n=51), and that dual hypermethylation was associated with typically adverse features, such as poor-differentiation. Despite slight inconsistency, abnormal genetic and epigenetic events associated with CDKN2A, including hypermethylation, are generally considered pro-carcinogenic, although via proliferative and anti-apoptotic mechanisms, rather than invasive and migratory.

TRPM1 was an upregulated (+7.28) gene coding for a transmembrane cation channel found in the retina and associated with depolarisation of the synapse in response to light, particularly in night-sight[562]. TRPM1 is also expressed in melanocytes and is expressed inversely with the aggressiveness (invasiveness and metastatic potential) of melanomas[563]. Calcium (Ca^{2+}) remodelling is an observed event in cancers and thought to promote tumour cell migration, encourage proliferation, and support resistance to cell death; although it the role of cation channels is not well characterised in colorectal cancers[564-566]. As calcium is a critical initiator of progression through the cell-cycle as well as being important to multiple other cellular processes, increased cation channel expression might be assumed to promote cell proliferation. However, this is contrary to the observed effect in melanomas, although the upregulation of other TRP-family genes has been shown to occur in association with the development and progression of other cancers[567, 568]. There is no published evidence examining a relationship between aberrant methylation events and expression of TRPM1 in cancers. The observation that upregulation of TRPM1 in DLD-1 following exposure to AZA may be associated with a reduction in invasion *in vitro* corresponds to the inverse relationship of expression and invasion in melanomas.

ITGβ1 (integrin-β1) was also upregulated based on array data (+3.11). Integrins are a large group of cell surface receptors that bind a huge array of ECM ligands and help regulate the cellular processes that are required for multicellular organisms to function normally, but also to dysfunction in cancers[569]. ITGβ1 is one of the many integrin regulators of TGFβ1 which can, as previously discussed, act as both a tumour suppressor and promotor[483, 485, 570]. Integrin-dependent upregulation of TGFβ1 and its downstream targets has been demonstrated in several cancer types and results in an EMT-like progression, including in colorectal cancer[571]. In breast cancer, Allen demonstrated that integrin-dependent activation of TGFβ1 was a key indicator of malignant transformation to ductal carcinoma in situ, a process dependent on TGFβ1 induced upregulation of MMP2 and MMP9[572]. Integrins also function to prevent anoikis by maintaining contact with the ECM, a regulatory control that may be lost in cancers[569]. Upon loss of integrin binding a number of downstream pathways are activated, including PIK3AK, resulting in caspase-8 recruitment to the cell membrane and activation by non-liganded integrins[573]. In neuroblastomas this process may be prevented by hypermethylation of caspase-8, resulting in silencing, but methylation events are not otherwise well characterised in integrins and cancer[574].

5.5.1.3 Unchanged Genes

As discussed, some genes of interest on the array that mechanistically could be important to the EMT-like and EMVI processes have been shown to be up- and downregulated following exposure to AZA. Other genes have however not demonstrated any significant fold-change in expression, some of which might have been important oncogenes in the progression of colorectal cancer. Of particular note were APC, KRAS, and SMADs.

The central role of adenomatous polyposis coli (APC) and KRAS genes in the classical Vogelstein model of colorectal cancer development has already been discussed in **Chapter 1.3.1**, but it is important to note that expression of these genes was not affected by demethylation with AZA. DLD-1 cells are known to demonstrated APC truncation, although the downstream effects of APC on β-catenin phosphorylation (and therefore function) are not determined by a binary relationship[262, 575, 576]. With regards epigenetic events and APC, hypermethylation of CpG islands associated with APC is a frequently observed event in sporadic CRC, with a significant increase in frequency of methylation of APC observed as adenomatous polyps progress to early cancers. Two recent meta-analyses have however demonstrated that APC promoter methylation does not correlate to any pathophysiological feature (including grade and TNM stage) of colorectal cancers, other than being a marker for distinguishing cancer from healthy or adenomatous tissue[577, 578]. These results suggest that APC may be important as an early initiator of colorectal cancer by stimulating proliferation and cell survival, but that it does not significantly affect the relationships that govern interactions with and invasion of the ECM[119, 120].

KRAS expression was also not affected by demethylation with AZA. KRAS mutation in CRC leads to deactivation and subsequent negative-signalling in the MAPK-pathway leading to increased signalling and uncontrolled cellular proliferation[111]. This process is not methylation-dependent and DLD-1 cells are known to demonstrate KRAS G13D amino acid substitution as a result of an underlying mutation that is not methylation dependent. This is consistent with KRAS-mutated tumours *in vivo*, where mutations most commonly occur at codon 12 and 13 and occur as part of the microsatellite-stable adenoma-carcinoma pathway[579].

Another family of genes not affected by AZA exposure was the SMAD family of intracellular signalling proteins (specifically SMAD2 and SMAD4). As previously discussed in **Section 5.1.1**, SMADs are important in EMT-like and metastatic processes in colorectal (and other)

cancers as they act to induce transcription that is a negative control on cellular proliferation and migration in response to TGFβ interactions with the stroma[482]. The lack of change in expression of these genes despite the marginally significantly decreased expression of TGFβ (-2.08), suggests that their downstream transcriptional targets may also not be dysregulated by AZA demethylation, although this process is also heavily depended on a number of other co-transcription factors not examined by the array[580]. Furthermore, as SMAD expression is associated with SLUG/SNAIL dependent transcription and subsequent CDH1 (E-cadherin) expression, the observed decreased expression of CHD1 (-2.59) may not be dependent on SMADs.

5.5.1.4 Summary

In addition to the MMP family of genes discussed in the following section, a number of gene important to EMT-like and invasive processes were up- and downregulated following exposure to AZA. Contrary to what might have been expected given the increased ability of DLD-1 cells to invade and migrate following demethylation as demonstrated in **Chapter 4**, the expression of E-cadherin (CDH1), an important cell-cell adhesion protein, was downregulated. Similarly, genes with important roles in the development of colorectal cancer, including early EMT-like progression, remained unchanged, although the genetic mechanisms linked to aberrant expression of these genes (APC and KRAS) in cancer is not methylation-dependent, although methylation may be observed. A number of canonical intracellular signalling genes associated with cancers were dysregulated (c-MET, ITGβ1, IGF1) or showed no change (SMADs). These results are difficult to interpret as there are multiple downstream targets of these pathways, many of which act in opposition to each other as a homeostatic mechanism, and some even display pro- and anti-tumoural activity depending on the stromal environment and other factors. However, classical tumour-suppressor gene CDKN2A was significantly up-regulated by demethylation with AZA, as was TRPM1, another gene associated with favourable features in melanoma. Clearly there is a mixed-picture in terms of the balance of up- and down-regulated genes and those that may be hypothesised as pro- and anti-tumoural, and thus it is pragmatic to address the specific observations of the effects of AZA on DLD-1 cells when interreacting with and ECM-like substrate such as that encountered during the invasion assay. This might be best evaluated by examining the effects of AZA on the metalloproteinase group of genes on the PRC array.

5.5.2. Metalloproteinases & qRT-PCR

Based on array data, MMP and TIMP expression was significantly changed across the family (relative fold-change): MMP2 (-4.10); MMP11 (+2.54); MMP13 (+2.85); TIMP2 (-2.86); TIMP4 (+2.58). For each of the genes highlighted by the array, qRT-PCR data individually validated and showed statistical significance for the up- or downregulation observed in the array following exposure to 0.50uM AZA for 72hrs, except for MMP13 which only showed an inverse but statistically significant $\Delta\Delta C_T$ after 24hrs exposure. Exposure to RRx-001 0.50uM for 72hrs universally upregulated every MMP or TIMP in a manner that was inconsistent with the effects of demethylation with AZA, but these effects only reached significance for MMP2 and TIMP4. In the case of MMP2, exposure to RRx-001 upregulated MMP2 in a contrary manner to exposure to AZA ($p \leq 0.01$). Unfortunately, MMP9 could not be included in the analysis due to unreliable qRT-PCR data. These results are summarised in **Table 5.5**.

Table 5.5 Summary of RT^2 Profiler PRC Array and qRT-PCR data for MMPs & TIMPs.

	Array fold-change	24hrs AZA		72hrs AZA		72hrs RRx-001	
		$\Delta\Delta C_T$	*p*	$\Delta\Delta C_T$	*p*	$\Delta\Delta C_T$	*p*
MMP2	-4.10	0.50	NS	0.24	$p \leq 0.001$	4.79	$p \leq 0.01$
MMP11	+2.54	2.25	NS	3.26	$p \leq 0.01$	1.47	NS
MMP13	+2.85	0.70	$p \leq 0.05$	0.76	NS	1.31	NS
TIMP2	-2.86	0.87	NS	0.37	$p \leq 0.001$	1.22	NS
TIMP4	+2.58	1.45	NS	5.89	$p \leq 0.05$	2.94	$p \leq 0.05$

These results demonstrate that as well as having the biggest fold-change based on the array data, MMP2 and TIMP2 also had the most statistically significant expressional change following 72hrs AZA exposure, based on $\Delta\Delta C_T$ qRT-PCR, although not necessarily the biggest absolute change. The large observed $\Delta\Delta C_T$ in MMP11 and TIMP4 were less statistically significant due to a relatively high standard deviation of mean $\Delta\Delta C_T$ for both genes, although they were above the threshold for significance. The results for MMP13 demonstrated borderline significance for change in expression (actual figure $p=0.03$) at 24hrs exposure to AZA, but not at 72hrs, despite a minimal standard deviation from the mean $\Delta\Delta C_T$ for each exposure time. This result was also contrary to that expected from the array data and suggests

the likelihood of a weak or insignificant effect of AZA on MMP13 at that dose across those time-points.

The results for RRx-001 were in many ways inconsistent and divergent from those of AZA, although there was an increased expression of each gene examined. Only MMP2 and TIMP4 demonstrated statistically significant $\Delta\Delta C_T$, but the absolute value for expressional change in each of these genes was high. There was however a high degree of deviation in the mean $\Delta\Delta C_T$ values for most genes following exposure to RRx-001; even in the genes demonstrating significant expressional change and statistical significance, and thus the effects of this agent on this family of genes is probably highly variable. The variability on results is likely be the result of alkylation and free-radical generation being imprecise modes of action affecting multiple downstream processes, as opposed to AZA's specific epigenetic effect.

The data from this study indicate that MMP2, the primary proteolytic enzyme of type-IV collagen was significantly downregulated by exposure to AZA; corresponding with the observation that DLD-1 cell treated with AZA are less able to invade a type-IV collagen-rich ECM. However, simultaneous upregulation of MMP11 expression, which is itself a proteolytic inhibitor of alpha-1-antitrypsin (another inhibitor of proteases), may have increased overall non-MMP proteolytic activity via a double-negative effect. TIMP2 was also observed to decrease expression following AZA exposure, thus the inhibitory effect of TIMP2 on MMP2 has also been down-regulated, contrary to the observed effect of less-ECM degradation. TIMP4 expression on the other hand increased following exposure to AZA; an unexpected finding as this protein is not normally found in the colon. Although the reduced propensity of DLD-1 cells to invade the ECM correlated with decreased MMP2 expression, the other findings of differential MMP and TIMP expression following AZA exposure do not fit well into a unified theoretical construct of hypermethylation resulting in increased potential for invasion.

However, the relationship between expression and inhibition of MMPs and TIMPs is complex, and separating the homeostatic controls from direct demethylation is challenging[581]. The data from this chapter suggests that AZA significantly reduced the expression of MMP2 but also TIMP2, although in different proportions. However, whether the decreased expression of MMP2 is as a result of direct demethylation with AZA or a homeostatic mechanism in response to reduced expression of TIMP2, or *visa versa*, or novel expression of TIMP4, is not clear.

5.6 Conclusions

RT-PCR array for gene expression associated with the EMT-like and invasive / metastatic processes associated with EMVI identified a number of genes of interest that may mechanistically account for the decreased propensity of DLD-1 cells to invade following exposure to AZA, and for CIMP-high tumours to demonstrate worse clinicopathological features. The family of genes that showed the most global change in expression were the metalloproteinases and their associated inhibitors. The MMPs and TIMPs are important in the EMT-like and invasive processes of colorectal cancer due to their actions on the components of the ECM[503, 504]. qRT-PCR for these MMPs and TIMPs revealed a complex picture of increased and decreased expression; both temporally and in relation to known and putative demethylating agents, although 72hrs of exposure to AZA resulted in statistically significant expressional change in metalloproteinases and their inhibitors with low margins of error. Despite the observations of expressional change qRT-PCR does not elucidate the transcriptomal or homeostatic mechanisms responsible for the observed effects *in vitro*. This, primarily, is due to the complexity of MMP and TIMP regulation, and how epigenetic modification may influence these processes. **Chapter 6** will aim to explore these relationships in more detail.

Chapter 6

Metalloproteinase Homeostasis and Demethylation

6.1 Introduction

6.1.1 Metalloproteinase Homeostasis

The regulation and function of MMPs and TIMPs is complex, occurring at a transcriptomal, post-transcriptomal, and extracellular level[581]. At a transcriptomal level, MMPs are regulated by *trans* activators such as AP-1, PEA3, Sp-1, etc, in response to a large number of cytokines, growth factors, and integrins; primarily via MAPKs and wnt/β-catenin canonical pathways[582]. Snail protein, for instance, has been shown to induce multiple MMPs and reduce E-cadherin expression, based upon downregulation of β-catenin in a process associated with EMT and differential intermediate filament expression[583]. Despite the consistency of *cis* elements within the MMP family, functionally similar MMPs often display different combinations of elements that may be shared between functionally unrelated MMPs, facilitating *cis*-element grouping (Groups 1-3)[582]. The balance and distribution of *trans*-activators and *cis*-elements across the MMP family is believed to facilitate homeostasis; a fall in one *cis* group being compensated by the reciprocal increase in expression of the other as they both express MMPs with a similar class of action. For instance, MMP9 (Gelatinase B) is in *cis* group 1, whilst MMP2 (Gelatinase A) in in *cis* group 3. Loss of this reciprocal expression is thought to underlie the development of some cancers[584, 585].

Epigenetic events have also been identified in the transcriptomal regulation of MMPs and TIMPs, with both hyper- and hypomethylation (and histone modification) being implicated in the differential expression of several metalloproteinases[586]. In lymphoma cell lines it has been shown that pre-existing hypermethylation of MMP9 inhibits expression, an effect that can be overcome by demethylation with AZA[587]. MMP9 is itself important in the progression of tumours but has primarily been identified as a key factor in inducing angiogenesis by VEGF and the metastatic processes associated with MET, rather than EMT, whereas MMP2 is not critical in this function[588]. In **Chapter 5**, expression of MMP9 was not changed following exposure to AZA based on the array, but unfortunately qRT-PCR for MMP9 failed. In another study, however, knockdown of DNMT1 and DNMT3a by homologous recombination in

colorectal cancer cell line HCT116 induced MMP3 expression whilst not affecting MMP1 and MMP2 expression significantly, thus the role of methylation, and the manipulation thereof, in inducing and repressing MMPs is complex[589]. As well as promoter methylation, histone modification also has a role in the epigenetic regulation of MMPs and can act in tandem with DNA methylation to control the "inducibility" of MMPs. For instance, methylation-dependent chromatin restructuring, which was itself regulated by activation of the MEK-1/extracellular signal-regulated kinase and NF-κB signalling pathways, was found to be regulatory in inducible MMP9 expression[590]. The role of methylation and chromatin was also highlighted in the specific case of MMP2 regulation by Vincent, who reported that methylation does not directly regulate MMP14, MMP2, or TIMP2 expression in human placenta, although it may contribute to chromatin remodelling-dependent expression of the MMPs[591]. Similarly, Chernov indicated that DNA demethylation alone was insufficient to modify the expression of MMPs in number of cancer cell types but was important in a "multilayer epigenetic regulation" that also involved chromatin restructuring[592].

In addition to transcriptomic control, post-transcriptomal regulation also has a role in regulating the expression of gelatinase MMPs. Such activity includes the prolongation of the half-life of MMP2 and MMP9 mRNA as may be observed in activated fibroblasts, or in response to innate cortisol, integrins, or applied therapeutics in the case of other MMPs[593-596]. Translational regulation of MMP & TIMP proteins in the ribosome may also contribute to the overall phenotypic expression of gelatinase-related activity, and dysregulation at this level has been associated with some forms of autism[597, 598]. However, once gene transcription and protein translation has occurred, the primary method of regulating MMP activity is by specific inhibitors secreted into the stroma.

TIMPs are the specific inhibitors of MMPs (as well as some other ECM proteolytic enzymes) and classically are the primary regulators of ECM turnover through their direct inhibition of MMPs; inhibition thus resulting in a homeostatic shift towards EMC component deposition as opposed to degradation[581, 599, 600]. TIMPs therefore have an important role in healthy organogenesis and tissue regeneration but have also been associated with conditions characterised by maladaptive ECM turnover such as keloid, lung fibrosis, heart failure, and cancer[601, 602]. As Type-IV collagen is the primary component of the basement membrane, much of the focus of research has been directed towards MMP2 and TIMP2, both in cancer and other conditions[603, 604].

MMP2 is inhibited by the wedge-shaped TIMP2 directly blocking its substrate binding site in a stochastic manner[605, 606]. As well as its role as an inhibitor, TIMP2 also auto-antagonistically activates MMP2 from its zymogen by co-association with membrane-type-1 MMP (MMP14)[602]. The regulation of MMP2 activity in the stroma is therefore based upon the concentration of MMP2, activation by MMP14, and the balance of the dual roles of TIMP2: the MMP2/TIMP2/MT1-MMP axis[592]. MMP14 was not assessed on the array or in qRT-PCR in **Chapter 5**. The role of TIMP2 and membrane-type MMPs in modulating MMP2 remains controversial. Some authors report that the activating/inhibitory relationship is dependent of the induced over-expression of activating membrane-type MMPs by fibroblasts in the stroma adjacent to early tumours, with tumour cell expression of activating factors being a characteristic only of more advanced tumours[607]. The activation and secretion of MMPs has also been suggested as a role of pro-tumoural tumour-associated neutrophils; a mechanism potentially underling the observations that the ratio of neutrophils to lymphocytes can have prognostic implications for colorectal cancers[608, 609].

Like MMPs, most TIMPs have multiple transcriptional control sites and respond to a range of signalling pathways; TIMP2 includes a TATA box at -30 bp, as well as a Sp1, two AP-2 sites, three PEA3s, and an AP-1[610]. Methylation events in the regulation of TIMPs are not well characterised, and the overarching narrative of the MMP/TIMP literature is that TIMPs play a poor second-fiddle to their better studied counterparts. There is, however, some evidence that TIMP2 has dual CpG islands in its promotor region, and that the balance of methylation at these islands has an important regulatory role in transcription[592].

As well as MMP2 and TIMP2, the array and qRT-PCR data also highlighted significant changes to the expression of TIMP4, an inhibitor of metalloproteinases which may also be important in cancer[611]. TIMP4 is a less well characterised inhibitor than TIMP2 that has also been shown to inhibit MMP2 by interaction with MMP14[612, 613]. However, unlike TIMP2, TIMP4 has no role in activating the MMP2 zymogen[614, 615]. TIMP4 has also shown activity against MMP9, and thus is considered as a less-potent but broader-acting inhibitor of Gelatinases, although its normal expression is limited to the heart, brain, and kidneys. One of the reasons for the limited distribution of TIMP4 is the differential expression of initiator sites in the promotor region; which demonstrates sites for myogenin, GATA and Ets binding but lacks the AP1 or AP2 sites characteristic of other TIMPs and MMPs[616, 617]. Although elevated

levels of MMP4 have been identified in a number of different cancers (ovarian, head & neck, and pancreatic adenocarcinomas), there is little evidence for its dysregulation in colorectal cancers[611, 618].

Clearly the regulation and function of MMPs and TIMPs is complex, occurring at a transcriptomal, post-transcriptomal, and the extracellular level[581]. In many circumstances TIMP and MMP genes are transcribed simultaneously, though the specific subset of MMPs and TIMPs depends on the external stimulus or internal genetic or epigenetic factor promoting or limiting expression. Once secreted into the stroma, the action of MMPs is dependent on the balance of activation and inhibition, although the relationship between MMPs and their corresponding TIMPs is not binary, and in many cases likely to be multifaceted. There is however an established relationship between MMP2, TIMP2, & MMP14 (MT-1 MMP), and potentially an emerging role for TIMP4 in this axis.

6.1.2 siRNA Knockdowns

When investigating the function of a gene and its coded protein a key tool to the investigator is the ability to prevent that gene being expressed, thus the function can be analysed by its absence. This is the principle behind *knockdown* technology, which can occur at the transcriptomal or post-transcriptomal levels. Pharmacological inhibition of target proteins is fundamentally different in terms of the phenotypic effects as the protein has already been synthesised so it is still present in the cell or microenvironment and its degree of function determined by the pharmacokinetics of the inhibitory agent[619]. At a genetic level, knockdowns are divided by their level of action; either to prevent transcription (as in the cases of CRISPR/Cas9 or TALE/TALEN) or post-transcriptional (such as RNA interference)[620]. However, the degree of knockdown is rarely 100%, meaning that a limited expression is usually apparent despite application of the technology due to a number of factors, including the binding saturations or transfection efficiency of the method used or cellular heteroploidy. The degree of knockdown and its reversibility will determine partly which method is applied, but also the more rudimental considerations such as speed, complexity, local expertise, and cost of each technology[620]. Note that *knockout* technology (mice and, increasingly, rats) is different in that it describes otherwise fully functioning animals that have had genes completely removed or replaced at a stem cell level prior to embryologic development, and therefore completely lack a particular gene[621].

At the post-transcriptomal level, short interfering RNAs (siRNAs) and their cousin the short hairpin RNA (shRNA) are a well-established method of preventing RNA transcription and thus gene knockdown[622, 623]. siRNA knockdown describes a process where RNA-targeted RNA oligonucleotides (20-25bp) bind intracellular proteins (following transfection) to form an RNA-induced silencing complex (RISC) that blocks translation of partially-complementary RNA sequences and degrades highly-complementary RNA sequences[624, 625]. As translation is prevented no protein is synthesised. siRNAs demonstrate less off-target effect than other knockdown technology, such as phosphorothioate S-DNA knockdowns, whilst being acceptably specific in terms of the sequence-specificity and thus gene of interest targeting. Concerns regarding the activation of the innate immune system by siRNAs is relevant only to their application in experimental systems or therapeutics involving live organisms with an intact immune system[626].

In an siRNA model dsRNA must be delivered into the target cell, necessitating penetration of the cell membrane. This may be achieved by three methods; transfection, electroporation, or viral-vector. Transfection involves the use of cationic liposomes, or other positively charged microparticles (such as polymer nanoparticles), that interact favourably with the negatively charged cell membrane to deliver siRNAs. This technique has the benefits of being cheap, reproducible, and effective at delivering siRNAs to most cell types, although it has a low efficiency in *in vivo* models[627]. Electroporation describes a process whereby an electrostatic field is applied to cells which transiently disrupts the cell membrane allowing passage in of siRNAs, but also the passage out of cell contents. This method has some advantages, including the potential for targeted siRNA delivery in vivo, but has a higher risk of toxicity and cell death than transfection[628, 629]. Viral-vector methods rely on engineered viruses, typically lentivirus but also retrovirus or adenovirus, to deliver siRNAs intracellularly[630]. Viral vectors have the benefit of being able to be applied to whole-organism systems, and may have a future role in delivering siRNA based therapeutic to hard-to-reach targets, such as the central nervous system in Huntingdon's Disease[631]. As such, siRNA-based technologies are increasingly being regarded as potentially viable therapeutic systems and are established in the *in vitro* manipulation of cells to investigate the knockdown-phenotype[632-634].

6.1.3 Immunocytochemistry & Protein Analysis

In **Chapter 5** the expression of genes was investigated by assessment of the relative amounts of mRNA that had been transcribed from DNA, thus demonstrating the relative transcription of the genes of interest (identification and quantification). Other methods of investigating gene expression examine the protein end-products of transcription via protein-specific methods; specifically, immunocytochemistry (ICC) and Western Blotting (WB) for proteins of interest. These techniques have the benefits of relatively rapidly demonstrating quantifiable changes in protein expression and can act as a verification of qRT-PCR.

ICC exploits protein (or polypeptide) specific ligands (primary monoclonal antibodies (mAbs)) that bind the protein of interest and can then be detected by fluorescence microscopy, either directly if they primary antibodies are themselves conjugated to a fluorophore (direct immunofluorescence), or via a secondary antibody that targets the primary and is itself conjugated to a fluorophore (indirect immunofluorescence (IIF))[635, 636]. This method allows the detection of specific proteins and is able to facilitate in localising the target within the cell. As techniques in ICC have evolved the sensitivity and specificity of primary antibodies has improved, as has the stability of the fluorophore[637]. The combination of fluorescence techniques with electron microscopy have further refined the level detail available to investigators examining the intracellular distribution of proteins[638]. ICC does, however, have limitations as a process; specifically, that as the cells being studied have to be fixed and made permeable to the mAbs; cells are thus not live during assessment by standard techniques and dynamic changes are therefore more challenging to study. Also, as cells require fixing and washing, ICC is limited in its ability to examine the extracellular distribution of secreted proteins, although it is able to determine intracellular stores of secreted proteins and proteins undergoing synthesis[639]. As with all laboratory investigations, it is important to use appropriate controls in ICC experiments to avoid confounding reults[640].

Western Blotting is a well-established and widely-used semiquantitative or quantitative technique for examining specific cellular proteins[641]. WB can be applied to samples as limited as a single cell and only a few μg of total protein, but care must be taken when processing samples during this multi-stage process to avoid erroneous results[642]. Like ICC, WB relies on specific mAbs binding target proteins and then a tagged secondary mAb facilitating visualisation, this time on polyacrylamide gels. Once protein-specific bands can be visualised a semiquantitative assessment of protein abundance can be made by densitometry or be used

in combination with fluorescence techniques[643]. Despite the multi-step process, WB does has the advantage of being able to assess the quantity of extracellular proteins as well as intracellular proteins, unlike ICC, and therefore offers a more complete examination of the effects of expressional modification[644].

6.2 Aims & Objectives

This chapter will examine the effects of demethylation with AZA on MMP and TIMP protein expression by two methods; immunocytochemistry and Western Blotting. These investigations will serve not only to aid in understanding the distribution of these proteins, how then interact, and how this is affected by demethylation, but also to cross-validate the results of the qRT-PCR. siRNA was also applied to DLD-1 cells in control and in conjunction with AZA exposure and the same assessment of MMP and TIMP proteins made; providing an insight into the homeostatic mechanisms of MMP and TIMP at a protein rather than transcriptomal level. Due to the divergence of the qRT-PCR results between AZA and RRx-001, and the unknown mechanisms of its actions, no further investigation will be conducted with RRx-001.

6.3 Methods

DLD-1 and HFL-1 cells were cultured in control conditions according to the protocols described in **Sections 2.3** and **4.3.1.2**. Modification in culture protocols for siRNA knockdown (KD) and exposure to AZA are described in the relevant sections. HFL-1 were used as a positive control for the expression of MMPs & TIMPs.

6.3.1 siRNA Knockdown & AZA Exposure

The siRNA KD protocol employed in this assay indicates that, following a successful transfection, treated cells should be ready to assay 24-72hrs following treatment. However, because the effects of AZA require cell division to become evident, as demonstrated by the 72hr exposure producing significant effects on qRT-PCR whereas 24hrs exposure did not, the manufacturers protocol was modified to facilitate longer AZA exposure. As transfection occurs in a serum-free medium, the transfection reagent removal and 1x complete medium replacement method was employed, rather than the addition of 2x medium to transfection reagents. This was to avoid toxicity associated with transfection and to facilitate AZA exposure. KDs were performed against TIMP2 and TIMP4 and with a scrambled siRNA control.

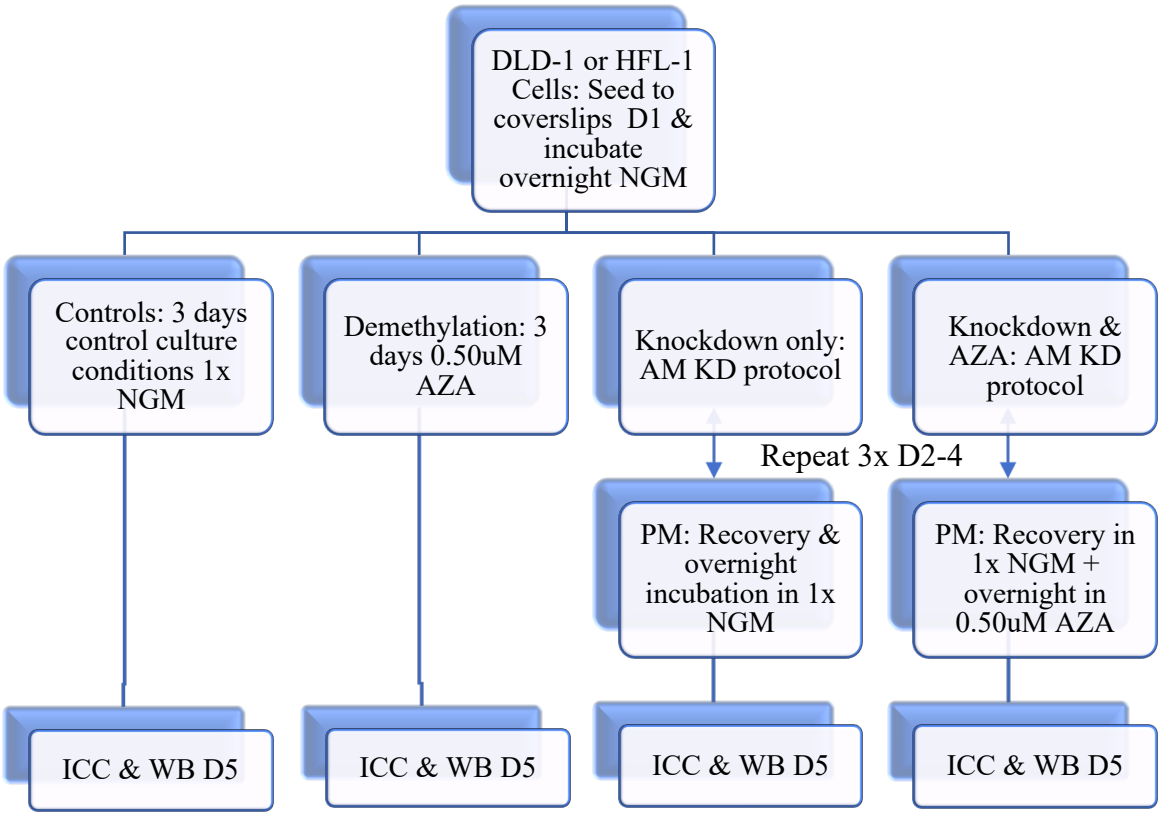
DLD-1 and HFL-1 cell lines were cultured according to maintenance protocols and prepared in normal (1x) antibiotic-free growth medium at a concentration of $3x10^4$ cells ml^{-1} (coulter counter assessment). Glass coverslips were sterilised in ethanol for 2 minutes and allowed to air dry in the bottom of a 6-well plate; three coverslips per well. Cells were then transferred to the wells containing coverslips allowed to adhere overnight. This technique facilitated both the production of control, AZA, and KD cells for subsequent ICC (cell on coverslips) and for protein analysis by WB (cells in the gaps). Three experimental arms were run: control, KD only, and KD with AZA.

All solutions (A&B) were prepared in accordance with the manufacturer's protocols for siRNA transfection. 100µL of the siRNA Duplex Solution (Solution A) was added directly to 100µL of the dilute Transection Reagent (Solution B) per well and mixed by pipetting and incubated at room temperature for 30 minutes. Normal growth medium then was removed from the wells by pipette aspiration and the cells washed once with 2ml of Transfection Medium which was then aspirated. 800µL per well siRNA Transfection Medium was added to the siRNA Transfection Reagent mixture and mixed gently by pipetting. 1ml total volume per well of

complete transfection and siRNA KD preparation was then overlaid onto the washed cells. Cells were then incubated for 6 hours in standard incubator conditions.

Following incubation, the transfection and KD preparation was removed and replaced by 1x antibiotic-free normal growth medium (containing serum) and the cells allowed a recovery-period of 2 hours. Following recovery, growth medium was removed and replaced with fresh medium for all wells, with the addition of 0.50uM AZA for treatment wells (fresh medium only for KD only) and incubated overnight. The entire process of transfection, recovery, and treatment with AZA or control was repeated for three consecutive days to allow effective demethylation with AZA. Following the final overnight incubation, the cells were assayed by ICC or protein analysis as described below. **Figure 6.1** demonstrates a flow chart of treatment protocols for siRNA KD and AZA cell culture.

Figure 6.1 Flow-chart demonstrating treatment algorithms for siRNA KD and AZA cell culture of DLD-1 and HFL-1 cells. NGM = normal growth medium; D1-5 = Day 1-5.



6.3.2 Immunocytochemistry & Immunofluorescence

An indirect immunofluorescence (IIF) method was adopted for all ICC studies. Working concentrations of each primary and secondary mAb was determined by means of a serial dilution and probe test. Serial dilutions of primary antibodies in PBS and 1.5% normal blocking solution were 1:50, 1:100, 1:200, 1:500; and 1:1000 and 1:2000 for secondary antibodies. Primary and secondary antibodies are listed in **Chapter 2**, **Table 2.4**.

Cells (DLD-1 and HFL-1; control, AZA, KDs, & KDs + AZA) were grown on coverslips according to the appropriate protocols as described in **Section 6.3.1**. Cell-covered coverslips were then removed from growth medium using sterile forceps and scalpel-blades and then washed twice in PBS by submersion to ensure removal of phenol-red, and then fixed by submersion in 3.7% paraformaldehyde (PFA) in PBS at 4^{o}C for 10 minutes. This was a deviation from the manufacturers protocol (recommending methanol, acetone, or formalin fixation), but 3.7% PFA is equivalent to 10% formalin and is thus considered interchangeable[639]. Following fixation coverslips were kept wet and rinsed in PBS 3 times and then permeabilised by incubation for 3 minutes in a solution of 0.1% Triton X-100 in PBS. Coverslips were then rinsed 3 times with PBS and then incubated in 10% normal blocking agent (3% BSA in PBS) for 30 minutes at room temperature. Following blocking, coverslips were then washed 3 times in PBS by submersion for 5 minutes. Coverslips were then replaced in 24-well plates and the primary antibody applied to completely cover each slide. The plate was sealed with biofilm to prevent drying and the cells incubated overnight at 4^{o}C.

Following primary antibody incubation, coverslips were washed 3 times in PBS by submersion and then incubated for 60 minutes at room temperature with the appropriate Alexa Fluor® 488-conjugated secondary antibody (mouse or rabbit to match the primary mAb) diluted in PBS with 1.5% normal blocking serum. Incubation with the fluorophore-conjugated secondary mAb was conducted in a darkened room with the plate wrapped in tinfoil to prevent degradation of the fluorophore. Coverslips were then washed 3 times in PBS by submersion for 5 minutes and immediately mounted to slides with VECTASHIELD mounting medium with DAPI nuclear stain (Vector Laboratories, Burlingame, CA, USA). Following incubation at 37^{o}C for 10 minutes, slides were imaged on the Metafer Automated Slide-Scanning platform (fluorescence intensity assessment: RAPIDSCORE) and Olympus BX51 Fluorescence Microscope (high-resolution images for cellular localisation of signal). Microscope settings were controlled for laser power and detector gain.

6.3.3 Western Blotting

Total protein was prepared by gently pipetting excess growth medium off cells cultured in a 12-well plate so that cells remained in a thin film of medium containing their secreted extracellular proteins. Plates were kept on ice throughout protein extraction and an ionic detergent method of cell membrane disruption was employed, exploiting the sodium dodecylsulfate and sodium deoxycholate constituents of RIPA Buffer[645]. Cells were then scraped from the bottom of their wells with a sterile scalpel until fully detached and then resuspend in 200µL ice-cold RIPA Buffer supplemented with 2µL of protease and 2µL of phosphatase inhibitors. The cells and RIPA buffer were mixed thoroughly by pipetting and transfer to pre-chilled microcentrifuge tubes and incubated for 5 minutes at 4°C. Cells were then lysed by vortexing thoroughly and then centrifuged for 10 minutes at 12,000rpm in a centrifuge pre-cooled to 4°C. The supernatant was then transferred to a fresh microcentrifuge tube and stored at -80°C until required for WB.

The Bio-Rad DC Protein assay kit was used to determine the protein quantity in each sample. All reagents were prepared as per kit instructions and all consumables were supplied by BioRad (except Tris buffers and gel ingredients). A protein standard of Bovine Serum Albumin (BSA) was diluted in RIPA buffer at 5 concentrations plus a negative control (0 to 2.5 mg/mL). Aliquots of 5µL of each protein sample or standard curve was then added to wells of microtiter plate (3 technical replicates). 25µL of working Reagent A was then added to every well plus 200µL of Reagent B and the plate was agitated for 15 minutes. The POLARstar Omega Microplate reader was then used to determine the protein concentration in each well, based on absorbances at 750 nm relative to the standard curve.

The proteins in the solution were the separated according to their molecular weight using the SDS-PAGE technique[646]. Two densities of gels were prepared for use as a stacking gel (4%) and a resolving gel (10%). Gels were hand-cast by the same method described in **Section 3.3.6** but with different recipes and the additional step of 0.5mL of isopropanol being added to the top of each gel to ensure that it was level. Gel recipes are listed in **Table 6.1**. The gels were then placed into an electrode assembly kit filled with running buffer.

Table 6.1 Recipes for stacking (4%) and resolving (10%) gels. Listed quantities make 2 gels.

	4% Stacking Gel	**10% Resolving Gel**
30% Acrylamide	650µl	5ml
ddH2O	3ml	6ml
1.5M Tris	1.25ml	3.75ml
1.0M Tris	50µl	150µl
10% SDS	25µl	75µl
TEMED	5µl	15µl

Proteins were thawed to room temperature and diluted at a 1:1 ratio with Laemmli buffer and then sonicated and heated at 95°C for 5 minutes. The first two wells of the gel were loaded with 8µL of dual-colour standard and biotinylated standard, respectively, and then a total of 40µg of each protein sample was loaded into subsequent individual wells. Gels were the run at 160 volts for 5 minutes (until the samples had reached the end of the stacking gel) and then 120 volts until the dye front approached the base of the resolving gel.

Immunoblotting was then performed to transfer the proteins onto PVDF membranes for antibody-probe analysis. One Immuno-Blot PVDF membrane per gel was activated by immersion in 100% methanol until translucent. All materials required for the protein transfer cassette, including fibre pads, filter paper, gels and membranes were equilibrated for 10 minutes in transfer buffer pre-cooled to 4°C. The transfer cassette was then assembled closed tightly to ensure that no air bubbles were present between the gel and the membrane. Electroblotting was performed at 400mA for 1h at 4°C. Following transfer, membranes were carefully removed from the transfer cassette using forceps and briefly washed in TBS/T wash buffer.

The PVDF membranes were then blocked for 60 minutes in 5% BSA at room temperature and then washed with TBS/T. Membranes were then incubated at 4°C overnight in 8mL of primary antibody solution diluted in blocking buffer to 1:1000. The same set of primary antibodies for MMP2, TIMP2, and TIMP4 employed in IIF were used for WB as they are all recommended for use in both processes by the manufacturer, and are listed in **Chapter 2**, **Table 2.4**. Membranes were then washed in TBS/T washing buffer 4 times each for 5 minutes with strong agitation. A second blocking process was then carried out at room temperature for 60 minutes

and with 1:1000 times diluted horseradish peroxidase conjugated secondary antibody (anti-mouse or anti-rabbit) for chemiluminescent detection. Membranes were again washed in TBS/T washing buffer 4 times each for 5 minutes with strong agitation.

Visual analysis of the protein quantities was then carried out the ImmunoStar Chemiluminescence Kit (BioRad). A 1:1 ratio of luminol/enhancer and peroxide solution was applied over the membrane and the ChemiDoc XRS (BioRad) system used to assess and quantify the relative band intensities on the membrane. Following imaging, the membrane was then stripped of the bands of interest using a stripping buffer (Thermo Scientific) at room temperature for 10 minutes with strong agitation, followed by washing 4 times for 5 minutes with a gentle agitation in TBS/T solution. A primary antibody for β-actin was then applied to the membrane (pre-mixed laboratory consumable supplied by a colleague) and the membrane re-incubated overnight at 4^{o}C before being washed, blocked, and imaged using the ChemiDoc XRS as described above. This visualisation using the beta-actin was then used to normalise the absolute protein quantities for MMP2, TIMP2, and TIMP4.

6.3.4 Statistics and Data Presentation

Results for change in abundance are described in terms of relative change in target protein abundance from the control based on either change in fluorescence or chemiluminescence. Control abundance will always be 1.0 with no standard deviation. Results of <1.0 indicates relative downregulation and >1.0 indicates relative upregulation. If absolute values for baseline are 0.0 (no protein detected) then the control abundance will still be 1.0, and subsequent results returning no protein detection will remain as 1.0. In the case of WB, protein abundance will also be controlled against β-actin abundance to provide internal normalisation of bands using ChemiDoc XRS. Standard descriptive statistics will be employed as appropriate and a Student's-T test used to assess any significance between two data-arrays. Cut-off for significance is set to p=0.05.

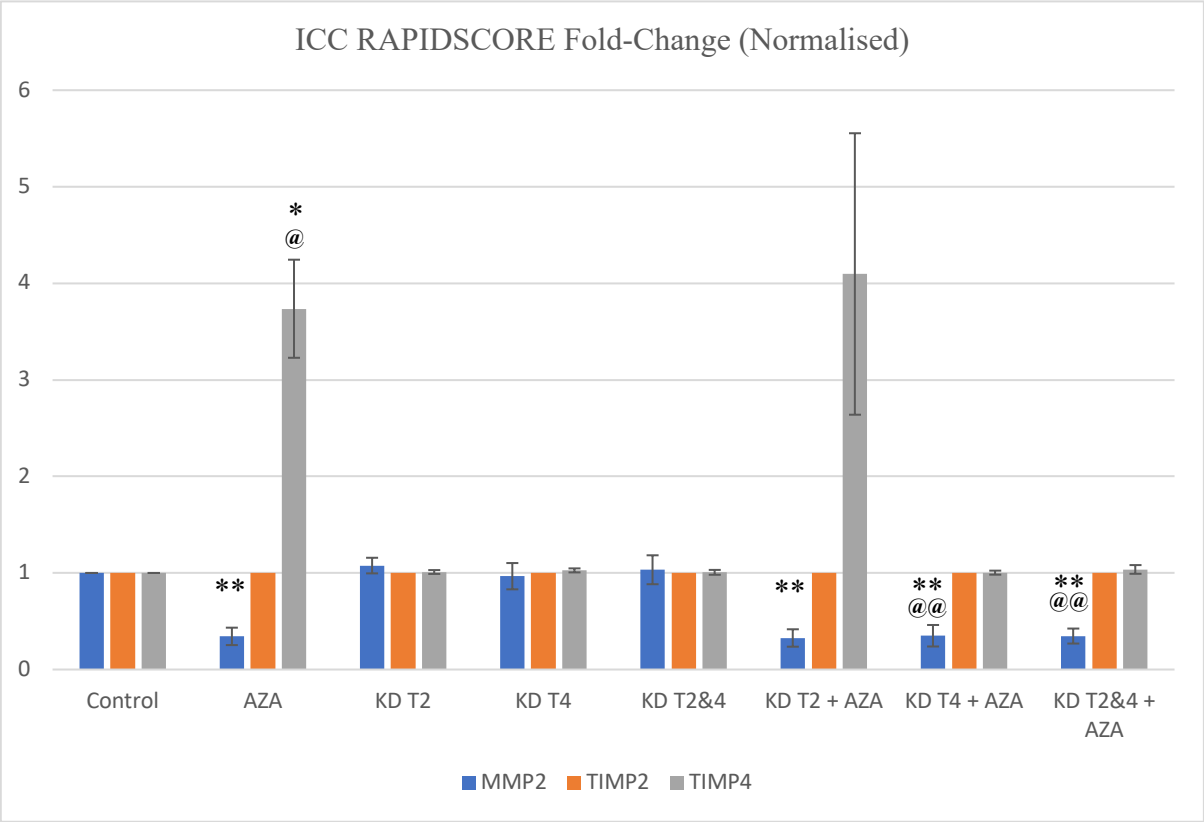
6.4 Results

Protein abundance was assessed by indirect immunofluorescence (IIF) for MMP2, TIMP2, and TIMP4 following the 4-arm exposure protocol described in **Section 6.3.1**. These proteins were selected as their corresponding genes were most significantly changed by AZA exposure based on array and qRT-PCR data in **Chapter 5**, and the MMP2-TIMP2-MMP14 axis is important in regulating degradation of Type-IV collagen rich extracellular components. Unfortunately, some technical and methodological issues limited the results gathered in this section of the investigation, although meaningful results were still recorded. Firstly, although HFL-1 cells were able to be maintained in normal culture medium in control conditions they did not survive the transfection process adequately, meaning that although data could be collected for their baseline MMP2 and TIMP expression, the relative effects of AZA and KD could not be assessed. This technical issue was unexpected as HFL-1 cells are generally easy to culture and have been shown to tolerate siRNA transfection targeting their metalloproteinase activity in a number of pervious studies[647, 648]. Specific issues relating to ICC and WB will be mentioned in the relevant results sections and explored further in the **Discussion**. Raw proteomic data is provided in **Appendix IV**.

6.4.1 Immunocytochemistry and Immunofluorescence

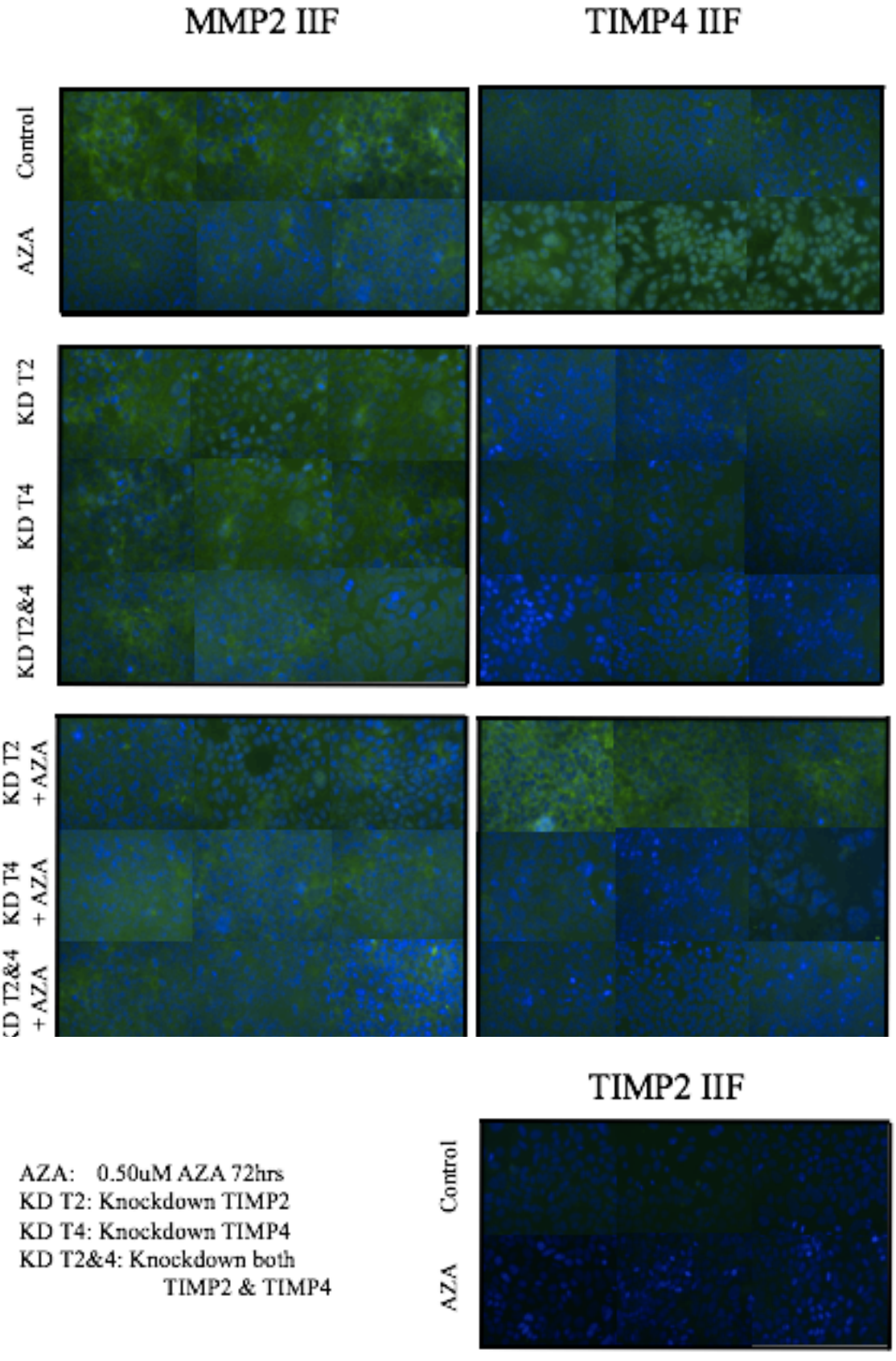
All treated samples underwent successful fixation, permeabilization, and primary and secondary antibody conjugation. There was not an apparent high-degree of cellular dehydration during fixation, consistent with the use of PFA[649]. Control HFL-1 cells demonstrated appropriate fluorescence when probed with each primary mAb an a manner consistent with the Human Protein Atlas (HPA) indicating adequate permeabilization with Triton X-100 and appropriate probe-access to crosslinked proteins[650]. A significant methodological limitation of this element of the experimental design is that MMP2 protein is only normally weakly present inside colonic cells and TIMP2 and TIMP4 are usually undetectable as they are almost exclusively extracellular, although significant upward expression of any protein may be detectable immediately following intracellular synthesis and before secretion, as should any decrease in MMP2. Results are presented graphically in **Figure 6.2** and Metafer images illustrating fluorescence are presented in **Figure 6.3**.

Figure 6.2. Relative fluorescence RAPIDSCORE for MMP2 and TIMP2 normalised to control fluorescence of 1.0. Error bars are standard deviation from the mean corrected fluorescence. [Δ mean fluorescence from control* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. ΔΔ mean fluorescence between target proteins@ $p \leq 0.05$; @@ $p \leq 0.01$; @@@ $p \leq 0.001$]



Note that the relative fluorescence is normalised to control and does not represent absolute values, thus TIMP2 and TIMP4 values are controlled to an expression of close to zero and subsequent negative fluorescence will return a result of 1.0 and standard deviation will be low. Demethylation with AZA resulted in a *de novo* expression of TIMP4 as well as a reduction in MMP2, but that KD of TIMP4 in association with AZA did not prevent the MMP2 response, therefore reduction in MMP2 expression is not dependent of the presence of TIMP4. The lack of significance observed in the response of TIMP4 to KD of TIMP2 and AZA is due to the high degree of deviation in individual TIMP4 RAPIDSCORE values (precise figures for Δcontrol and ΔΔMMP2 were $p=0.067$ and $p=0.052$, respectively, indicating a high degree of trend).

Figure 6.3 Representative immunofluorescence (IIF) images for MMP2, TIMP2, and TIMP4 in DLD-1 cells following exposure to control, AZA, knockdown, or combination protocols.



Control ICC for baseline signal from each of MMP2, TIMP2, and TIMP4 was consistent with that expected based upon the HPA; specifically, that MMP2 demonstrated a detectable signal and that TIMP2 and TIMP4 were undetectable. This was due to limited abundance of these TIMPs within the cell. Control ICC for DLD-1 cells demonstrated the presence of intracellular MMP2 but neither TIMP2 nor TIMP4. This was consistent with the predicted result of the HPA which predicted no intracellular TIMPs, but not with the array or qRT-PCR data that suggested that there was a baseline expression of each gene, although this was much higher for MMP2 and TIMP2 than TIMP4.

Following exposure to 0.50uM AZA for 72hrs, ICC demonstrated a relative decrease in MMP2 expression (Δ=0.34 ±0.09; p=0.006), no change in TIMP2 expression, but an increase in TIMP4 expression (Δ=3.74 ±0.51; p=0.011). These results are partially consistent with the qRT-PCR data, confirming the relative change in abundance/expression of MMP2 and TIMP4, but not demonstrating any significant change in TIMP2. As baseline TIMP2 was negative, the expected decrease in protein abundance/expression would not be expected to be evident.

Following siRNA KD of TIMP2, TIMP4, or TIMP2 & TIMP4, DLD-1 cells demonstrated no change in abundance of MMP2 and no change to the lack of signal for TIMP2 and TIMP4. Scramble knockdowns did not result in any change in baseline signal for any target. Validation was provided by Western blotting, see **Section 6.4.2**.

When KDs were performed in association with treatment with 0.50uM AZA, the previously observed decrease in MMP2 following AZA exposure alone was preserved in each experimental arm (Δ=0.32-0.35 ±0.07-0.11; p=0.005-0.01). The increased expression in TIMP4 following exposure to AZA was also observed when anti-TIMP2 siRNA was employed in combination with AZA but did not reach statistical significance due to a high degree of deviation, although a strong trend was observed (Δ=4.10 ±1.46; p=0.07). This effect was lost when TIMP4 was knocked down. These observations confirm the effective KD of TIMP4 and also indicate that the increase in TIMP4 observed with AZA is not dependent on TIMP2 downstream signalling (TIMP2 KD was confirmed by WB). The results also indicate that the reduction in MMP2 abundance is not dependent on TIMP4 protein abundance, as the effect is preserved when TIMP4 is knocked down.

Fluorescence microscopy images acquired using the Metafer platform are provided in **Figure 6.3** to illustrate the differing abundance of detected proteins. The quality of slide preparation and resulting image blurring prevented meaningful evaluation of the cellular distribution of MMP2 and TIMP4 and was thus abandoned.

6.4.2 Western Blotting

Protein abundance was observed for MMP2, TIMP2, and TIMP4 following the 4-arm exposure protocol described in **Section 6.3.1**. Unfortunately, a technical difficulty was encountered in the primary antibody probing or secondary conjugation for TIMP4 as chemiluminescence failed to detect any bands irrespective of experimental arm. Protein extraction and other processes for these samples were however adequate as β-actin did produce visible bands after stripping and re-probing. All results are illustrated in **Figure 6.4**.

Baseline expression of MMP2, TIMP2, and TIMP4 in HFL-1 cells was in keeping with the expected phenotype; with both MMP2 and TIMP2 detectable, although probing for TIMP4 failed. This is consistent with typical fibroblast activity and confirms that ICC results for TIMP2 are due to lack of intracellular protein, rather than no transcription or protein synthesis. As stated previously, HFL-1 cells did not tolerate transfection and thus only this baseline assessment produced reliable results. In DLD-1 cells, baseline abundance of MMP2 and TIMP2 was in keeping with that observed in the qRT-PCR data and matched that observed in HFL-1 cells. Detection of MMP4 failed.

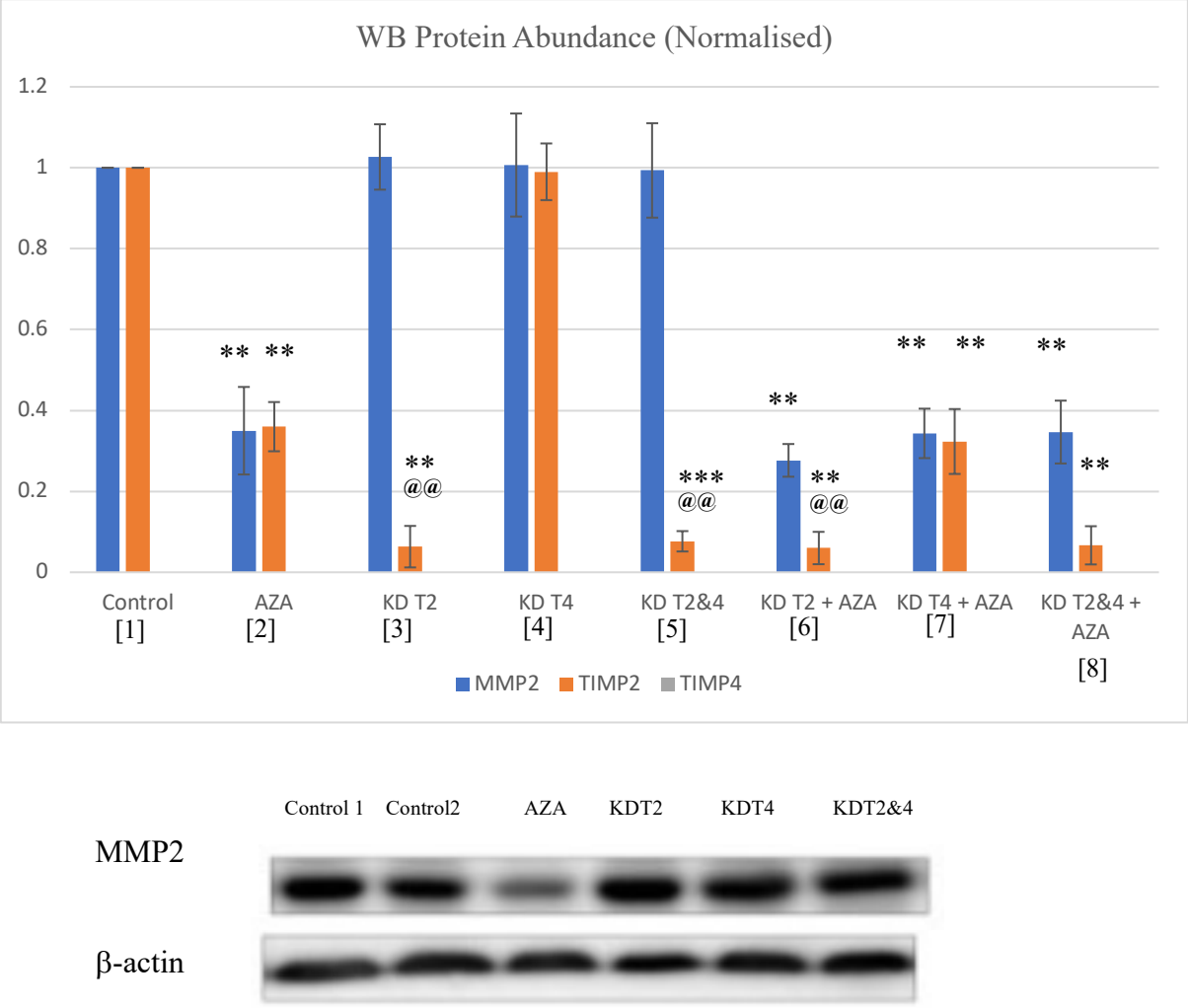
Following exposure to AZA, DLD-1 cells demonstrated reduced abundance of both MMP2 (Δ=0.35 ±0.11; p=0.009) and TIMP2 (Δ=0.36 ±0.06; p=0.003). These results are in keeping with the results of the array and qRT-PCR data. Although TIMP4 was not detectable by WB due to technical failure, increased abundance was previously demonstrated by IIF.

Following knockdown of TIMP2 there was a significant reduction in the abundance of TIMP2, although minimal amounts were still detectable, confirming effective but incomplete KD with siRNA (Δ=0.06 ±0.05; p=0.001). This was important as IIF had shown no change to TIMP2 as it was not detectable due to its extracellular location. KD of TIMP2 did not significantly affect the abundance of MMP2 (Δ=1.02 ±0.08; p=NS). KD of TIMP4 did not significantly affect the abundance of MMP2 or TIMP2, although combined KD of TIMP2 and TIMP4 produced a reduction in TIMP2 abundance consistent with TIMP2 KD alone.

Following exposure to AZA and KD of TIMP2 both a reduction in TIMP2 and MMP2 abundance was observed; the reduction in MMP2 being proportionate to that observed with AZA alone (Δ=0.27 ±0.04; p=0.001) and that of TIMP2 being proportionate to siRNA KD alone (Δ=0.06 ±0.04; p=0.001). There was also a significant difference between the reduced abundance of MMP2 and TIMP2 (p=0.009). KD of TIMP4 in association with AZA also produced a decreased abundance of both TIMP2 (Δ=0.34 ±0.06; p=0.003) and MMP2 (Δ=0.32 ±0.08; p=0.005) but was proportionate to that observed with AZA alone. Combined KD of TIMP2 & TIMP4 with AZA exposure resulted in a response identical to TIMP2 KD and AZA.

The most important findings based on WB data is that KD of TIMP2 alone did not significantly affect the abundance of MMP2, and that demethylation with AZA in combination with KD of TIMP2 produced a reduced abundance of MMP2 that was proportional to that of AZA alone. It can be inferred from these results that a reduction in TIMP2 protein, whether by demethylation or KD, is not necessary for the decrease in MMP2 following AZA exposure. Additionally, neither MMP2 or TIMP2 appear to be affected by TIMP4.

Figure 6.3. Relative protein abundance for MMP2 and TIMP2 normalised to control abundance of 1.0. Error bars are standard deviation from mean corrected abundance. A corresponding blot excerpt is shown to demonstrate abundance for MMP2. [Δ mean abundance from control* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. ΔΔ mean abundance between target proteins@ $p \leq 0.05$; @@ $p \leq 0.01$; @@@ $p \leq 0.001$]



Note the significant reduction in abundance of MMP2 and TIMP2 observed with AZA alone [2] ($p \leq 0.01$), and that there was an effective siRNA KD of T2 [3] ($p \leq 0.01$), but that this did not affect MMP2 abundance (p=NS). However, when TIMP2 was KD, MMP2's abundance was significantly reduced by AZA [6], indicating that the reduction in abundance of MMP2 in response to AZA is not dependent on the abundance of TIMP2. KD of TIMP4 had no effect on the abundance of MMP2 or TIMP2 in any treatment [4-5], or KD combination [7-8].

6.5 Discussion

The aims of this chapter were to assess the effects of demethylation on the protein abundance of MMP2, TIMP2, and TIMP4 by both an immunocytochemistry and Western Blotting methodology. Further to this baseline assessment, the knockdown of TIMPs singularly, in combination, and concurrently with AZA exposure was intended to provide insight into the homeostatic dependency of any relationship between MMP2 and TIMP abundance, and to isolate the effects of AZA at a transcriptomic level.

As previously discussed, the regulation of MMP and TIMP expression is complex and occurs at a transcriptomal, post-transcriptomal, and protein level; the balance of which determines phenotype[581, 600]. The results demonstrate that the reduced expression (qRT-PCR) and abundance (IIF & WB) of MMP2 observed with AZA is independent of the abundance of TIMP2 and TIMP4 and must therefore be assumed to be dependent on a direct demethylating effect at a transcriptomic level, or due to AZA's effect on another regulatory mechanism that is not dependent on these TIMPs. The results also indicate that the increase in expression of TIMP4 observed following exposure to AZA does not independently affect MMP2 or TIMP2 expression. Unfortunately, due to a number of technical and methodological shortcomings, cross-correlation between protein abundance studies was not possible, although the observed effects on MMP2, TIMP2, and TIMP4 were consistent with those observed in **Chapter 5**.

The expression of MMP2 in relation to methylation status has been reported to be variable across a number of different studies; some indicating that hypomethylation of MMP2 promotor sequences increased expression in breast cancer and glioma cell lines, and was in associated with an increasingly aggressing phenotype[546, 592, 651]. However, whole-genome pyrosequencing studies have confirmed the frequent occurrence of MMP2 CpG island hypermethylation in colorectal cancer, a finding which has been supported by clinical datasets[652, 653]. The findings in this study are in alignment with the whole-genome pyrosequencing studies. The divergent findings relating to the expression of MMPs and methylation status across tumour types is not unexpected; Couillard having previously shown that expression of MMPs (based on mRNA techniques) in response to demethylation was not only variable between MMPs in colorectal cell lines, but also that often the directly opposite effect was observed between different tumour types[589]. The implication of the acknowledgement that different tumour types express different patterns of MMP and TIMP methylation, and that they respond differently to manipulation of methylation at transcriptomal and phenotypic level, should not undermine the concept of

hypermethylation-associated upregulation of MMP2 being important in the pathogenesis of colorectal cancer. Breast cancers and gliomas are, after all, different cancer types with different cells-of-origin and a different phenotype; particularly considering the differences in phenotype expected between diffuse-type and solid-type tumours.

With regards to the potentially reciprocal transcriptomal control of MMPs, the findings of this Chapter do not help delineate MMP or TIMP response to demethylation based on promotor motif grouping[582]. The system proposed by Yan and Boyd suggest that MMP2 is a Group 3 MMP characterised by no TATA box and no proximal AP-1, instead demonstrating a number of GC boxes, AP-2, and STAT[581, 582]. However, TIMP2 shares GC boxes, STAT, and AP-2 sites with MMP2, but also has a TATA box and AP-1 as well as NF-κB sites. TIMP4 on the other hand, like MMP2, has no TATA box, but does have NF-κB, AP-1, and AP-2, although reduced GS sites. Due to the divergence in transcriptional response to AZA and the lack of clear association between this response and putative grouping based on promotor motifs, or indeed any specific promotor sites, no direct (motif demethylation) or indirect (motif activator effect) action of demethylation effecting MMP and TIMP transcription via one-or-other binding site or group can be identified. Furthermore, the mechanisms by which nuclear hormone receptors interact with MMP promotor motifs is complex and diverse, and do not necessarily correspond to neat binary relationships between co-active or counter-active MMPs and TIMPs, suggesting an additional layer of complexity in constitutive and induced transcriptomal regulation[654]. This is despite previous studies indicating that specific promotor motifs influence the balance between constitutive MMP2 and TIMP2 expression, although admittedly not in relation to methylation but instead to polymorphism[585, 655, 656].

As the promotor motifs in the promotor regions in MMP2 and the two TIMPs are highly overlapping but the effects of demethylation not convergent, promotor demethylation affecting transcription is not occurring at the identified activator motifs but may be occurring at another upstream site. CpG islands and differential methylation in approximation to MMPs and TIMPs have been associated with a variety of diseases, including cancer, stroke, clotting disorders, and placental defects[657-659]. Although epigenetic events have previously been identified in metalloproteinase transcription, including the MMP2/TIMP2/MT1-MMP axis, CpG islands are not well characterised in relation to MMPs and TIMPs, and these genes do not contribute to the classical CIMP classification systems, although some of the ADAM group of proteinases are included in a limited number panels (see **Chapter 1**, **Table 1.2**)[592, 660, 661].

As well as DNA methylation, transcriptomal regulation of MMP2 and TIMP2 by chromatin restructuring has also been shown to be important in cancers including colorectal cancer, and indirect effects of DNA demethylation affecting chromatin structure cannot be excluded (such as DNA methylation-dependent deacetylase expression)[189]. However, as AZA specifically affects DNMT activity, the effects observed in this study are not due to direct modification of histone methylation as might be observed following dysregulation with enhancer of zeste homolog 2 (EZH2) in breast and ovarian cancers[662, 663].

At the post-translational level, over-expression of TIMP2 has been associated with suppression of wnt/β-catenin and subsequent upregulation of proliferative and pro-tumoural cell activity in ovarian cancer mediated via ubiquitination[664]. The effects of low TIMP2 were overcome with application of lithium in this study, which re-activated the previously down-regulated signalling pathway. The results in this chapter are to a degree contrary to this study, as the reduction of MMP2 following AZA exposure was not dependent of the abundance of TIMP2, and therefore not reliant on a post-translational process dependent on TIMP2. This was demonstrated by the persistence of the change in abundance of MMP2 following TIMP2 KD. The independence of MMP2 abundance and TIMP4 abundance also indicates a lack of dependency between these two proteins.

When considering the results of this chapter however, it should be acknowledged that there are several limitations that prohibit the transference of the hypothesis that hypermethylation-related upregulation of MMP2 drives invasiveness of DLD-1 cell *in vitro*, an effect that can be reversed by direct demethylation with AZA, to the *in vivo* colorectal cancer. One limitation is that the investigations in this Chapter were somewhat limited by both the methodological construct (probing for extracellular proteins with intracellular ICC techniques) and technical failures (failure of chemiluminescence for MMP4). There was however consistency across each of the elements of this Chapter and the findings of **Chapter 5**'s qRT-PCR and thus they are, together, confirmatory.

With regards the detection of TIMP2 (or failure thereof) in the intracellular cytoplasm, although primarily an extracellular protein, other authors have reported being able to detect TIMP2 with standard techniques and the product literature supplied with the kit demonstrates intracellular detection in HeLa cells following a standard methanol-fixation technique[665, 666].

The use of intracellular transport blockers such as monensin or Brefeldin-A was avoided due to increased complexity and the potential for confounding (monensin has been shown to interfere with wnt signalling in colorectal cancers), especially in the siRNA arms of the experiment[667, 668]. The use of novel single-cell dynamic intracellular inducible protein analysis was beyond the remit of this study[669].

The lack of detectable bands against TIMP4 in the WB analysis was also disappointing. Protein extraction was successful as bands were demonstrated for β-actin, but no chemiluminescence was detectable when probed for TIMP4. The first possibility is that the primary antibody is not effective in the WB assay, although it worked adequately in ICC and is supported for both of these uses in the product literature[670]. The second possibility is that the conjugation to the secondary antibody was not effective due to a cross-reactivity issue or other fouling. The host for the primary antibody used for TIMP4 was rabbit and thus specific anti-rabbit secondary was acquired for ICC, but the anti-rabbit horseradish peroxidase secondary for WB was not ordered specifically for use with this primary (laboratory stock, different manufacturer), and thus there is a possibility it was not compatible with the primary or was degraded. Unfortunately, due to time and resource constraints, the re-running of the whole assay (including cell culture, siRNA transfection, etc) was not possible, and since results from ICC for TIMP4 were in accordance with the qRT-PCR data, was also deemed unnecessary.

Another consideration is that the experiments conducted in this and the previous Chapter were performed on cells not interacting with an ECM-like environment, as was the case for the invasion assays in **Chapter 4**[671]. When cells of any kind interact with the stroma their phenotype is changes by ligand activation of multiple pathways, and thus the expression of important genes of interest and translation of proteins will be markedly different from cells cultured on plastic or glass. These differences are likely to be augmented when cells are not only interaction with the ligands of the ECM but with other cell types, as would be encountered in the *in vivo* tumour microenvironment[672]. It should be noted, however, that the initial array and RT-PCR data in **Chapter 5** was based on expression following exposure to AZA on cells not cultured in an ECM-like environments (e.g. on Matrigel), thus the effects of demethylation were not confounded by other changes in cellular phenotype in response to ECM ligands. Furthermore, the methods employed in this Chapter do not model the heterogeneity observed in in vitro tumours; the assessment of gene expression by mRNA and protein techniques having

been performed for total culture extracts. This homogeneity, or apparent homogeneity, is unlikely to be representative of the tumour environment *in vivo*[399, 477].

Despite these issues an overarching narrative is emerging linking demethylation of DLD-1 cells in vitro, reduced transcription and protein abundance of MMP2 and TIMP2, and a reduced propensity of demethylated cells to migrate and invade. These finding are summarised in **Table 6.2**.

Table 6.2 Summary of findings relating to expression of genes of interest and the methylation status of DLD-1 cells in vitro. Direction of arrows denotes direction of regulation, X denoted fouled reaction. Figures denote statistically significant relative fold change[@], ΔΔCT*, or relative abundance[$].

	ARRAY		qRT-PCR		IIF		WB	
	AZA[@]		AZA*		AZA[$]		AZA[$]	
MMP2	↓	-4.10	↓	0.24	↓	0.34	↓	0.35
MMP9	↔		X		-		-	
MMP11	↑	+2.45	↑	3.26	-		-	
MMP13	↑	+2.85	↔		-		-	
TIMP2	↓	-2.68	↓	0.37	X		↓	0.36
TIMP4	↑	-2.58	↑	5.89	↑	3.74	X	

6.5 Conclusions

The results of this Chapter demonstrate that exposure to AZA reduces the protein abundance of MMP2 & TIMP2 and increases TIMP4, as demonstrated by ICC and WB methodologies. These findings are in keeping with those observed based on expressional array data and individual qRT-PCR (the results for exposure to RRx-001 were divergent and prone to statistical deviation). AZA has also been shown to decrease the abundance of MMP2 & TIMP2 protein and increase TIMP4 protein in a manner that was not dependent upon the presence of the counterpart proteins, suggesting AZA is directly affecting the transcription of each gene rather than via a post-translational homeostatic mechanism within the MMP2/TIMP2/MT1-MMP axis. Furthermore, as the motifs in the promotor regions of MMP2 and the two TIMPs are highly overlapping but the effects of demethylation not convergent, demethylation affecting these sites is less likely to be responsible for the observed change in expression which is more likely occurring at another upstream site, such as a CpG island.

MMP2 and TIMP2 are the major gelatinase and respective inhibitor responsible for degradation of Type-IV collagen in the basement membrane. Demethylation with AZA results in a reduced propensity for DLD-1 CRC cells to migrate and invade *in vitro*. The relative decrease in MMP2 and dysregulation to gelatinase expression and abundance may be able to account for the reduced ability of AZA exposed DLD-1 cells to invade in an EMT-like model of early cancer development and metastasis that is analogous the processes involved in EMVI. These findings do not however directly translate to the *in vivo* process of early cancer progression and EMVI due to the relative crudity of the *in vitro* modelling (lack of ECM, stromal cells, microenvironmental hypoxia, etc). Investigating the effects of methylation status on gelatinase expression and tumour histopathological characteristics (including EMVI) will be the overarching aim of **Chapter 7**.

Chapter 7

Extramural Vascular Invasion (EMVI) in Rectal Cancer and the *In Vivo* Expression of Metalloproteinases & their Inhibitors.

7.1 Introduction

Chapter 3 introduced the concept of extramural vascular invasion (EMVI) in rectal cancer and how this radiological and histopathological feature of rectal cancers is associated with poor prognosis and the requirement of adjunct therapies. The diagnostic challenges in detecting the presence of EMVI pre-operatively, thus presenting the possibility of neoadjuvant therapies in an effort to improve outcomes, were also discussed. **Chapter 3** also examined the association between EMVI and the CpG Island Methylator Phenotype (CIMP), demonstrating a statistically significant association between the epigenetic events characterised by CIMP and the presence of histopathologically demonstrated EMVI, although the presence of the CIMP phenotype did not translate to poorer clinical outcomes[258].

Subsequent Chapters in this thesis have examined the relationship between the biological effects of methylation on colorectal cancer cell lines *in vitro*, and how manipulation of methylation can alter the phenotype. Demethylation with 5-azacytidine (AZA) has been shown to reduce the migratory and invasive ability of DLD-1 cells in association with dysregulation of metalloproteinases (MMPs) and their inhibitors (TIMPs) gene expression and protein abundance. Investigation with putative demethylator RRx-001 produced less consistent results. The reduced ability of cancer cells to migrate and invade is important *in vivo* as these abilities are crucial to the processes defining the epithelial-to-mesenchymal transition (EMT) and metastasis, processes analogous to EMVI and early rectal cancer progression[476, 490].

Novel associations between EMVI, MMP & TIMP expression, CIMP, and clinicopathological features and outcomes may, therefore, be beneficial in facilitating the diagnosis of EMVI or in risk-stratifying rectal cancers: Can metalloproteinases serve as biomarkers in rectal cancer?

7.1.1 Metalloproteinases as Biomarkers

As already introduced in **Chapter 5**, MMPs and TIMPs have an established role in the progression of many cancers, specifically relating to their ability to remodel the extracellular matrix as early cancer cells interact with the cellular and acellular components of the stroma[505, 510]. The MMP2/TIMP2/MT1-MMP axis has itself already been identified as a potentially important mechanism for early cancer progression in colorectal cancer and has been associated with a worse Duke's Stage[511, 512]. However, the translation of benchtop observations into the manipulation of MMPs in *in vivo* therapeutics has been limited in success, suggesting additional layers of complexity in the relationship between MMP expression and cancer biology[514]. This is also reflected in the failure of MMPs and TIMPs to be adopted as diagnostic and predictive biomarkers.

Aside from MMPs' and TIMPs' mechanistic role in cancer progression, their potential as biomarkers has been investigated via a number of methods for over 25 years. In 1991, Levy examined the mRNA expression of MMP2 in 18 colorectal cancers and compared expression to adjacent normal mucosa, demonstrating an increase in expression in 13 cases (72%)[511]. Immunohistochemical examination of a further 70 specimens, including 30 adenomas, demonstrated a statistically significant increase in staining for MMP2 (expressed as a percentage of positively stained cells) in Duke's A/B over adenoma, and Duke's C over Duke's A/B (all $p<0.001$). However, contrary to these findings, Ring (1997) demonstrated that MMP2 and MMP9 were not correlated with worse Duke's stage or to survival, but that TIMP2 was inversely proportional to worse Duke's stage and tumour differentialtion[512]. Interestingly, IHC demonstrated that MMP9 expression in tumour-associated macrophages was significantly associated with poorly differentiated tumours in this study ($p<0.05$) and MMP2 demonstrated a trend ($p=0.08$). In another series of 71 colorectal cancers, Zeng showed that MMP9 expression (assessed by mRNA Northern blot hybridisation) was associated with synchronous distant metastasis ($p=0.004$) and worse Duke's Stage ($p=0.008$), and that dichotomisation into MMP9-high and -low groups was a predictor of shorter disease-free and overall-survival ($p<0.0001$ and $p<0.0002$, respectively)[673]. Circulating concentrations of MMP1 have also been found to be associated with and predictive of metastasis and Duke's C stage by other authors, based on ELISA-like assessments[674]. What this early research best illustrates is that there is huge variability between studies in terms of the association with disease stage and prognostic significance of individual MMPs and TIMPs, and that these associations have been found to be variably demonstrable between different investigations.

Very few studies have investigated the role of MMPs and TIMPs specifically in rectal cancer. This is largely due to colorectal disease being considered one biological entity in much of the literature despite a growing appreciation of the longitudinal differentiation between molecular subtypes along the colon[202]. In 2007, Schwandner examined 94 rectal cancers by IHC for multiple MMPs and TIMPs, finding that MMP2, TIMP2, and MT1-MMP were associated with the depth of invasion of the tumour, but not with differentiation, overall tumour stage (UICC), pre-operative CEA level, or nodal status[675]. None of the studied MMPs or TIMPs was associated with OS or DFS, although pre-operative CEA was significantly associated with both (both $p<0.01$). However, Tohoku found that only MMP9 was associated with worse survival ($p=0.03$) and nodal involvement in a series of 64 rectal cancers examined by IHC, whereas MMP2, TIMP2, and TIMP3 bore no association[676]. Again, the divergence in finding between studies is highlighted, and note that neither study examined any association between MMPs & TIMPs and EMVI.

More recent reviews of the utility of MMPs and TIMPs in the diagnosis and staging of colorectal cancer have highlighted the challenges of the multiple different techniques in quantifying transcription, translation, and activity of MMPs; and have attributed the failure of this group of mechanistically important proteins to translate to useful biomarkers on the reliability and variability of different methodologies[677]. Although a variety of methodologies are employed, the assessment of proteases in cancer diagnosis and prognosis has been blighted by inconsistencies in findings, leading to few such genes and associated proteins being used as viable biomarkers[604, 678]. These issues are not unique to colorectal cancer and MMPs have also failed to become viable biomarkers in urological, breast, and other cancers despite extensive investigation. There are however ongoing efforts to build consensus that MMPs and TIMPs are potential diagnostic and prognostic biomarkers, with a particular focus on MMP2 and MMP9, although none has yet been adopted[682, 683]. Even given promising predictive values of MMPs in symptomatic patients (specifically circulating MMP9 assessed by ELISA), the focus on screening and diagnostic tools aimed at unselected populations has led to the predominance of faecal testing and other risk-stratification tools that direct patients towards colonoscopy (or equivalent)[679-681]. No studies have examined any association between metalloproteinases and EMVI from the perspective of being a potential biomarker that may aid in identifying at-risk patients, and certainly none in the context of DNA hypermethylation events.

7.1.2 Tissue Microarrays

Tissue microarrays (TMA) are a high throughput method of assessing intact tissue at a genomic, transcriptomic, or proteomic level[684]. The fundamental principle of a TMA is the precision micro-sampling of tissue blocks and side-by-side mounting onto a limited number of slides so that multiple sections can be exposed to the same assay and then assessed rapidly[685].These techniques allow assessment and storage of multiple tissue samples and are an evolution of the pre-existing "sausage" techniques[686]. The primary time and resource advantage of TMAs is that once representative specimens have been selected the process may then be automated and the number of new slides, as well as consumables employed to process them, dramatically reduced. Samples are then mounted sequentially on slides without individual labelling, thus keeping input samples in the same order as the mounting is crucial[687].

The pairing of TMA slide preparation with modern techniques in IHC facilitates internal control as multiple specimens can be exposed to the same probing and resolving process symaltaneously[688]. The biological principles in IHC are essentially the same as those exploited in ICC and IIF (see **Chapter 6**) but are employed on an intact tissue sample, thus facilitating an appreciation of the tissue-distribution of staining[689]. This advantage is therefore beneficial in examining potential intra-sample heterogeneity (such as tumour heterogeneity) and also the distribution of targets across different cell types when compared to ICC, although is less powerful than traditional full-slide assessment. TMAs have therefore become a standard and invaluable method of molecular profiling and can be combined with bioinformatic techniques to generate large datasets and meaningful insights into in cancer and other diseases[690, 691].

When assessing TMAs, as in other forms of IHC, it is important to adhere to standards of reporting and interpreting data. Standard methods for ensuring the reliability of data is dual specimen selection and reporting by pathologists, but also techniques in intensity and distribution scoring are important[692, 693]. As such, the five-step process of *masking*, *examination*, *lesion parameters*, *scoring definitions*, and *interpretation consistency* advocated by Gibson-Corley is recommended[694]. By employing these methods, a semi-quantitative output can be achieved that is robust and meaningfully descriptive, avoiding vague descriptions of staining such as "weak" or "strong".

7.2 Aims & Objectives

This Chapter will investigate any associations between the histopathological and clinical features of resected rectal tumours and metalloproteinase expression and CpG-methylator phenotype (CIMP), with a particular focus on EMVI. The principle source for the histopathological and clinical data will be the database from which similar data was extracted and investigated in **Chapter 3**. Tissue microarrays will be employed to probe the expression of key MMPs and TIMPs in resected cancers and an inductive semi-quantitative methodology employed to cross-correlate against the clinical dataset and the observed effects of demethylation *in vitro.* The objective is to close the circle on a potential association between clinical outcomes in rectal cancer, EMVI, hypermethylation, and differential expression of MMPs & TIMPs.

7.3 Methods

7.3.1 Patient Selection, Data Collection, & Storage

Sixty rectal cancer patients were extracted from the prospectively maintained database as described in section **2.1.1** & **2.1.2**, and in accordance with the ethics laid out in section **2.1.3**. The sixty patients were selected to provide an even split between those patients demonstrating EMVI on the histopathology examination of their resected tumours. Patients were in all other ways unselected and otherwise drawn at random from the pool. As previously stated, patients were neoadjuvant therapy naïve, and exclusion criteria also included patients with hereditary or other identifiable predispositions to carcinogenesis, such as inflammatory bowel disease. All patients underwent index surgery between January 2010 and May 2013 at a single centre (ABMU, Morriston Hospital, Swansea, SA6 6NL, UK) by a team of surgeons participating in the Swansea Pelvic Oncology MDT. Tumour characteristics were defined as per the ACPGBI or Royal College of Pathology guidelines with respective relation to pre-operative (mTNM) or postoperative (pTNM) staging and other tumour characteristics (CRM, differentiation, EMVI)[35, 206]. Adjuvant chemotherapy was administered based on current guidelines and best-practice. Patient identifiable data was stored in keeping with the principles of NIHR Good Clinical Practice (GCP) and the UK Policy Framework for Health and Social Care Research[364]. For further specific detail of patient selection, data collection, and storage please refer to **Chapter 3**, **Section 3.3.1**.

7.3.2 Tissue Microarrays & IHC

TMAs were constructed by biomedical scientists of the Pathology Department of Singleton Hospital. Tumours were retrieved from storage and assessed by a consultant histopathologist to ensure representative areas of solid tumour were being sampled. Samples were anonymised after retrieval and mounting to TMA by means of dual-coding to remove any patient identifiable data from the data collection phase. Data was then re-coupled to patient clinical, histopathological, and CIMP datasets for analysis.

The Ventana BenchMark ULTRA IHC/ISH System (Roche, Basel, Switzerland) was employed to process TMAs as it fully automates the baking, deparaffinization, cell conditioning and staining, counter stain and titration processes. Each primary antibody was optimised for antibody concentration and incubation time and temperature by the automated BenchMark ULTRA System and checked against positive control (human liver) by biomedical scientists. Details of the primary antibodies are found in **Chapter 2** (**Table 2.4**), but each was

supplied by Abcam (USA) and chosen based on experimental requirement (for targeting MMP2, MMP9, MMP11, TIMP2, and TIMP4) and the expertise of the biomedical scientists (choice of supplier, variant of mAb, process optimisation, etc). Positive control was also mounted on the TMA to ensure internal validity of the staining process.

Unstained FFPE TMA sections (4um) were mounted onto glass slides according to standard protocol and baked for 60 minutes at 60°C. The slides were then barcode labelled and placed on the BenchMark ULTRA System. Complimentary Ventana reaction buffer was used throughout the process to rinse slides and all other consumables were drawn from existing histopathology laboratory stock that had previously been optimised for the equipment. Liquid cover slip solution and reaction buffer is applied to the slides before each step and after each wash. Dewaxing is carried out at 72°C with EZprep solution. Retrieval with CC1 (cell conditioner) was then be performed at 98°C for between 8 and 48mins depending on antibody. Pre-peroxidase inhibitor was added. 100µl of diluted (dilution determined during optimization) primary antibodies was titrated onto the slides and incubated for between 4 minutes and 2 hours (depending on the protocol determined during optimization of the antibodies) at either room temp, 40°C, 37°C or 42°C (depending on the protocol determined during optimization of the antibodies). OV HQ Universal linker containing secondary antibody was then applied and incubated for 8 minutes, followed by OV- HPR multimer for 8 minutes. Sections were then treated with OV DAB and H_2O_2 for 8 minutes before finally being incubated in copper for 4 minutes. An optional final incubation with OV AMP multimer was carried out depending on the antibody for 4-8 minutes. The slides were then be counterstained with Heamatoxylin for 8 minutes.

IHC staining was assessed according to a modified protocol based upon the recommendations set out in the Fedchenko review and with reference to the Gibson-Corley recommendations[692, 694]. Scoring was carried out by a panel of at least three expert observers comprising at least one consultant histopathologist. The remaining participants were the investigator and academic supervisor (clinical or non-clinical), or biomedical scientist or other parallel Doctorate co-investigators. Disputes were resolved by the consultant histopathologist who had the deciding opinion. TMAs were observed using light microscopy at 40-100x magnification and a consensus scoring system was adopted. The scoring system is set out in **Table 7.1** and the scoring proforma in **Appendix V**. The overall staining metric Mx was determined by multiplying the score for the proportion of tumour cells stained by the score for the intensity

of staining; giving a maximum score of 12. Multiplying the scores (as opposed to adding the scores) ensured that uniformly non-staining tissues scored 0 (p4 x i0 = 0, rather than 4), and that the score was powered towards the more objective proportion of stained cells rather than the subjective intensity of staining for those that did score[692]. TMAs were performed in triplicate for each tumour (patient) sampled and the metrics averaged. This method provides semi-quantitative data for non-parametric analysis.

Table 7.1 IHC scoring system for TMAs

Proportion of Cells	**p-Score**	**Intensity of Staining**	**i-Score**
None	0	None	0
<10%	1	Weak	1
10% – 50%	2	Intermediate	2
51% - 90%	3	Strong	3
>90%	4		
MAX Multiplication Score (Mx) = 12			

7.3.3 Statistical & Test Analysis

Multiplication scores (Mx) were for each MMP or TIMP (0-12) in sample triplicates and then averaged to provide the overall score, as described in **Section 7.3.2**. Scores were then categorised (Cat scores) according to the system set out below. Nominal categorisation was preferred as the data is discrete rather than continuous.

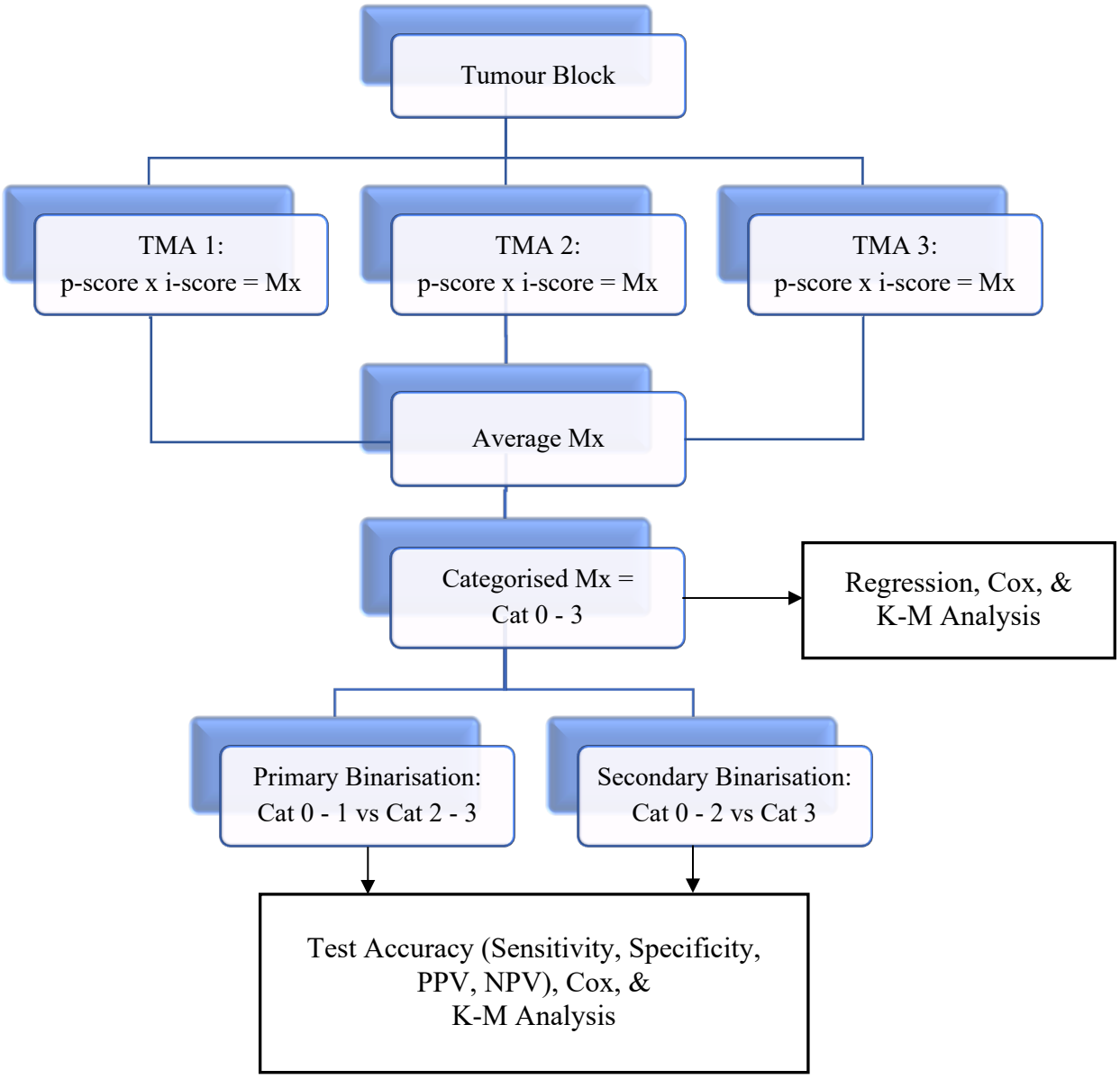
- Category 0 (Cat 0): Mx 0
- Category 1 (Cat 1): Mx 1 to 4
- Category 2 (Cat 1): Mx 5 to 8
- Category 3 (Cat 2): Mx 9 to 12

Categorisation is consistent with the methods set out by other authors in benchmark papers on methods in IHC[695-697]. Following categorisation, global assessment of association to EMVI was made for each MMP and TIMP by logistic regression and Cox proportionate hazard (time-to-event) modelling[698]. A survival analysis by Kaplan-Meyer method (survival-distribution function by Log-rank) was also estimated for each MMP and TIMP. Following global assessment, if association was indicated, test optimisation was performed by Category sub-analysis for sensitivity and specificity so that the optimal combination of categories could be constructed into the best test by binarisation. Binarised tests were then re-assessed for proportionate hazard and survival modelling. This process is summarised in **Figure 7.1**.

Pearson's correlation matrix was them employed to demonstrate association between CIMP and both MMP2 and EMVI in the sample population.

Data analysis was performed using the Microsoft Office Excel (v.16.12) and the XLStat Addinsoft (v.2017) plug-in (macro). Normalcy of data distribution was determined using a Kolmogorov-Smirnov test. If data were normally distributed, statistical significance was determined using Student's t-test for single comparisons or one-way ANOVA where more than one comparison was made. If data were not normally distributed, Mann-Whitney's test was used to compare unpaired means and Wilcoxon test used for paired data. Log-Rank (Mantel-Cox) tests were used where data was right-side skewed and censored. Comparison of expected frequencies was performed with two-tailed Chi Squared or Fishers exact test if observed events were less than 10. Survival curves were calculated using Kaplan-Meier estimate. The null hypothesis was rejected at the level of 5% ($p>0.05$).

Figure 7.1 Flow diagram of IHC analysis & outputs for each MMP or TIMP.



7.4 Results

7.4.1 Patient and Tumour Characteristics

There were 60 patients included in this study (30 patients with an EMVI-positive tumour and 30 with no evidence of EMVI). Forty-two patients were male (70%) and males were statistically more likely to demonstrate EMVI than females (p=0.005). The median age was 68 years (range 45 – 89 years), and patients were of a similar age in both groups. Nineteen patients had died at the time analysis was undertaken. Median follow up was 56.5 months (IQR 25 – 71 months). Mean overall DFS and OS were 28 and 33 months, respectively, but DFS was 26 months in the EMVI-positive group and 31 months in the EMVI-negative group (p=0.20). OS between EMVI-positive and -negative groups was 32 and 34 months, respectively (p=0.51). When differences between both groups were analysed between groups, patients with EMVI-positive tumours had more advanced pathological staging by pT, pN, and AJCC classifications (p=0.038, p=<0.0001, and p=0.001, respectively). Specifically, when the T-grade was compared against EMVI, pT1 and pT2 were significantly less frequently occurring events than others within the contingency table (each Fisher's exact text p<0.05). There was a corresponding association between EMVI-positive tumours and the need for adjuvant chemotherapy (p=<0.0001). There was, however, no statistically significant difference in CRM positivity, tumour perforation, or tumour differentiation (all p=NS). These findings are demonstrated as three-dimensional (3D) contingency tables in **Figure 7.2**.

7.4.2 Staining Characteristics and EMVI

TMAs for 60 tumour specimens were successfully constructed and stained against the targets MMP2, MMP9, MMP11, TIMP2, and TIMP4. All staining was successful and controlled against the human liver positive control except for MMP11 which failed to stain against tumour and control despite multiple attempts at automised and manual optimisation. Following percentage and intensity scoring, the multiplied metric (Mx) was calculated and tumour Categorised scoring (Cat) performed; these results are demonstrated in the frequency histograms in **Appendix VI.a**. Representative TMAs stained for MMP2 and TIMP2 are demonstrated in **Figure 7.3 a** & **b**. Logistic regression analysis of the Cat scores demonstrated a significant relationship between EMVI and MMP2 and TIMP2 (both p<0.0001). No relationship was discernible between EMVI for and Cat scores for MMP9 or TIMP4 (both p=NS). The receiver operating characteristic (ROC) analysis of global MMP2 Cat scores demonstrated an area-under-curve (AOC) of 0.947 indicting it is a good binary classifier.

However, ROC analysis for TIMP2 was less favourable (AOC 0.560). These results are also demonstrated graphically in **Appendix VI.b**.

Figure 7.2 3D contingency tables for EMVI against **a.** pT; **b.** pN; **c.** AJCC classification, and **d.** adjuvant chemotherapy. X^2 test for all contingencies $p<0.05$.

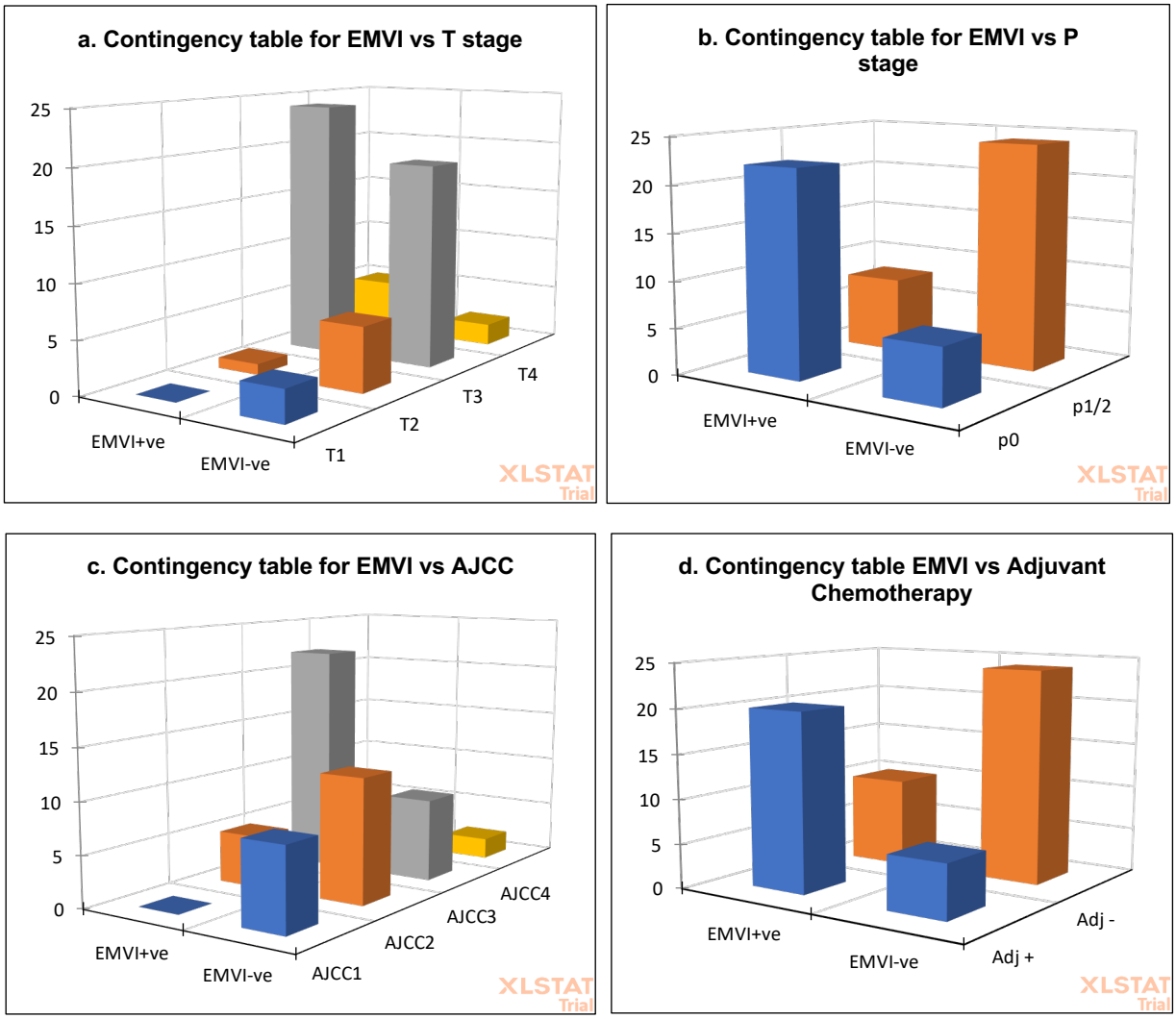
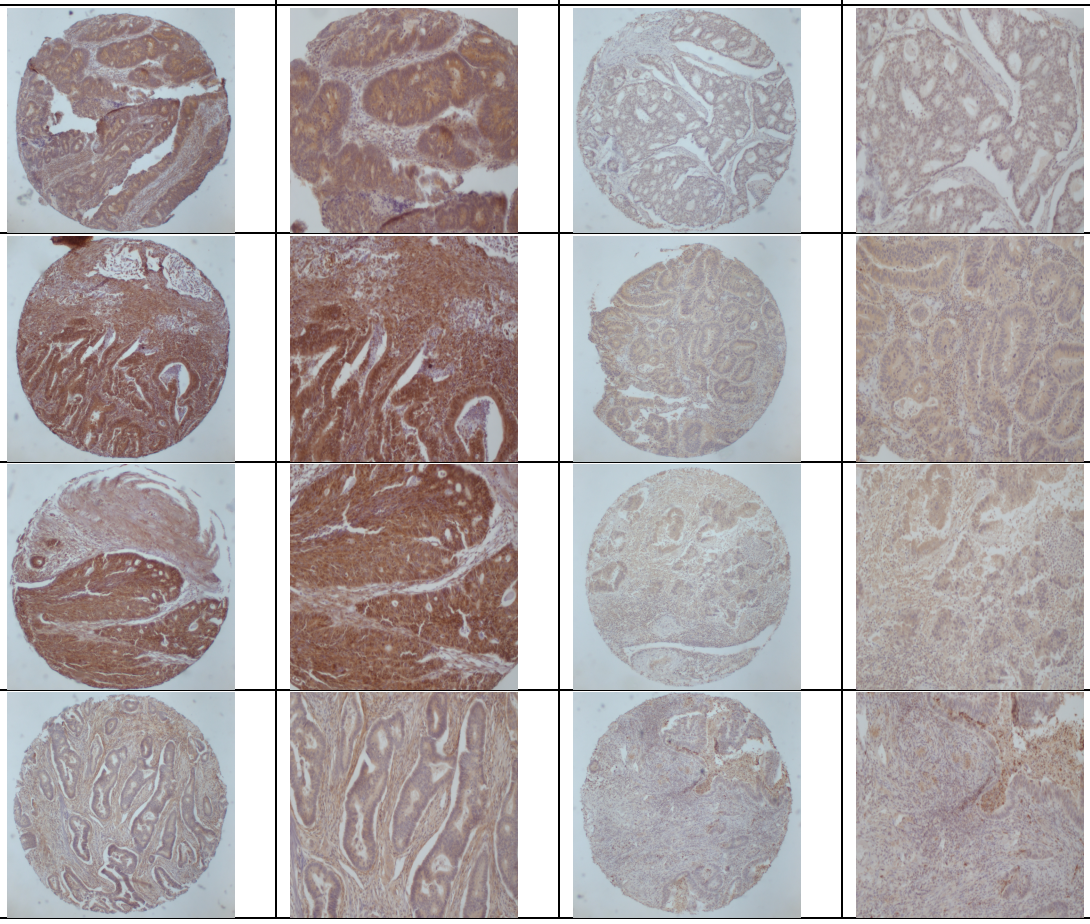


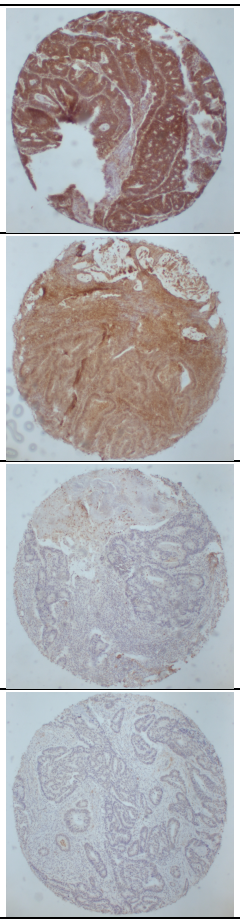
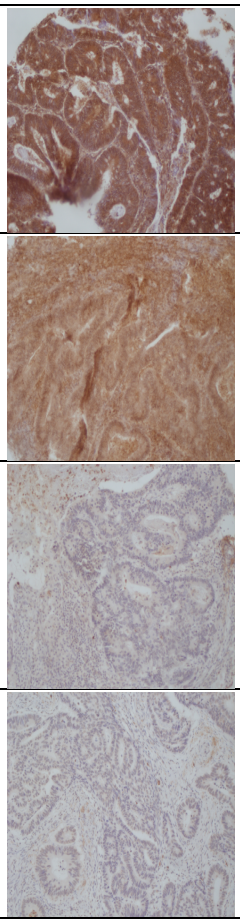
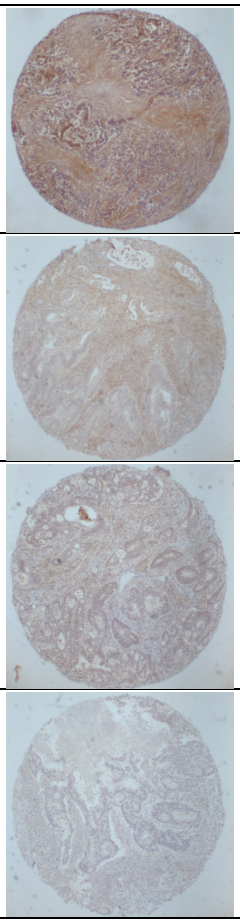
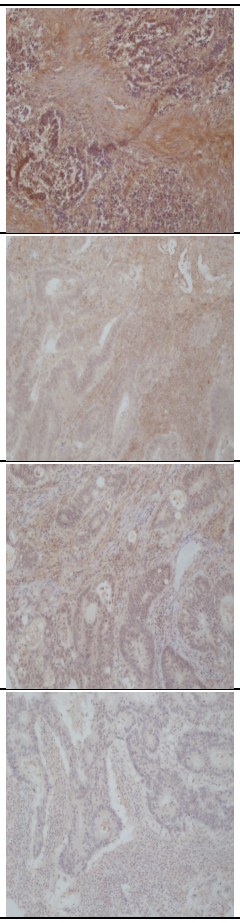
Figure 7.3a & &.3b. Selected representative light microscopy images at x4 and x10 magnification of IHC for MMP2 & TIMP2 in EMVI-positive and EMVI-negative tumours.

a.

	Selected representative light microscopy images at x4 and x10 magnification of IHC for MMP2 in EMVI-positive and EMVI-negative tumours.			
	EMVI-positive		EMVI-negative	
	x4	x10	x4	x10
MMP2				

Note the differential staining intensity between EMVI-positive and EMVI-negative tumours in this selected representative series. Following scoring categorisation, this differential staining was demonstrated to be significant (p<0.0001). Binarised scoring was then demonstrated to be predictive of EMVI-status and DFS and OS (see further results in this Chapter).

b.

	Selected representative light microscopy images at x4 and x10 magnification of IHC for TIMP2 in EMVI-positive and EMVI-negative tumours.			
	EMVI-positive		EMVI-negative	
	x4	x10	x4	x10
TIMP2				

Although staining intensity was associated with EMVI-status in this series (p<0.0001), TIMP2 was not found to be a predictive discriminator for EMVI, as was the case for MMP2. This series has been constructed to also highlight the differential degrees of staining proportion and intensity used to calculate the multiplication score, Mx, in the IHC analysis (top row = high score, bottom row = low score; see **Section 7.3.2**)[695-697].

7.4.3 Proportional Hazards in relation to EMVI and Staining Characteristics

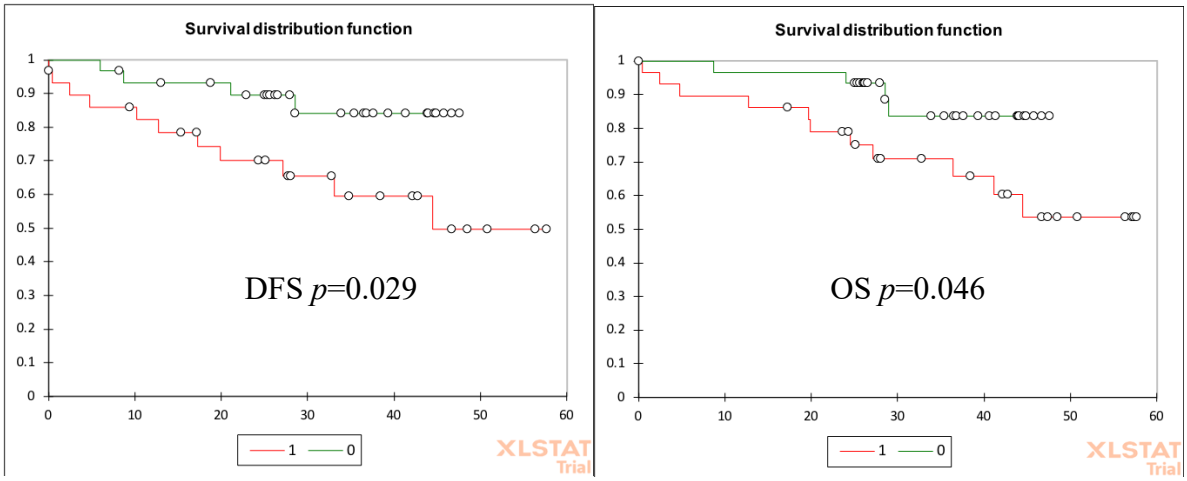
The Cox Proportionate Hazards Model was used to examine the proportionate risk of poor disease-free (DFS) and overall (OS) survival in relation to EMVI, and Global MMP2 & TIMP2 Cat scores. Cox modelling demonstrated that EMVI was significantly related to DFS and OS with Hazard Ratios of 3.32 (p=0.027) and 3.05 (p=0.043), respectively. The same analysis for MMP2 and TIMP2 demonstrated statistical non-significance for their risk relating to DFS and OS, (p=0.11 and p=0.15, respectively, for DFS and OS). These results are demonstrated in **Appendix VI.c** with the accompanying Log(-Log(SDF)) curves demonstrating similar slopes for each test, although there is a degree of left-shift for each and more so for EMVI, indicating that the risk-ratio is divergent, and hence it reaching significance for increased risk in this time-point model.

7.4.4 Survival Analysis

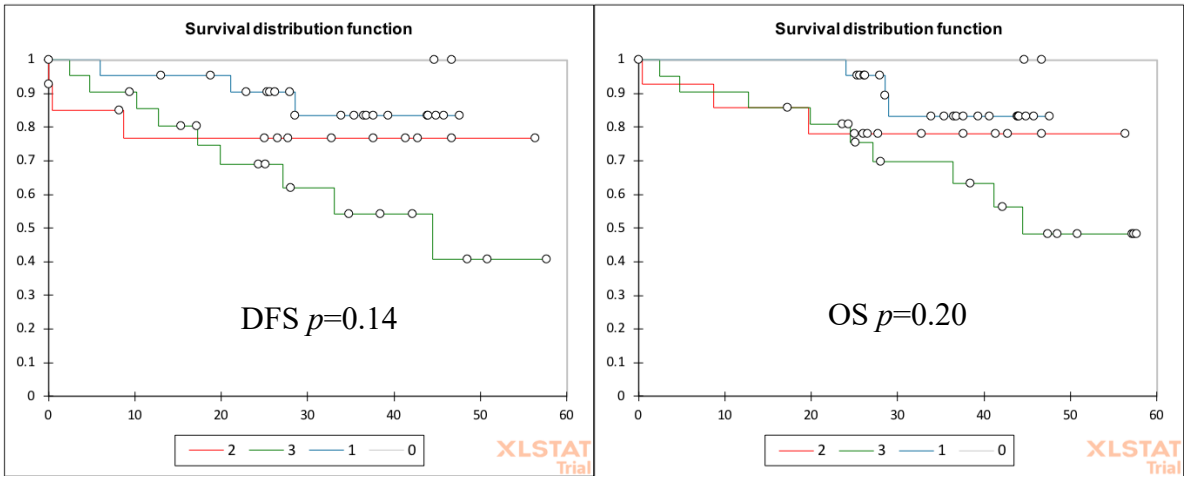
Kaplan-Meyer (KM) analysis was performed for DFS and OS in relation to EMVI, and Global MMP2 & TIMP2 Cat scores. This analysis showed that EMVI was significantly related to both DFS and OS (p=0.029 and p=0.046, respectively). Similar to the results demonstrated in Cox modelling, the KM analysis demonstrated non-significance for both MMP2 and TIMP2 when Cat scores were analysed as a group, (p=0.15 and p=0.20, respectively, for DFS and OS). These results are demonstrated in **Figure 7.4**.

Figure 7.4 Kaplan-Mayer Survival Distribution Functions for Disease-Free (DFS) and Overall Survival (OS) assessed on EMVI and categorised staining (Cat) scores for MMP2 & TIMP2.

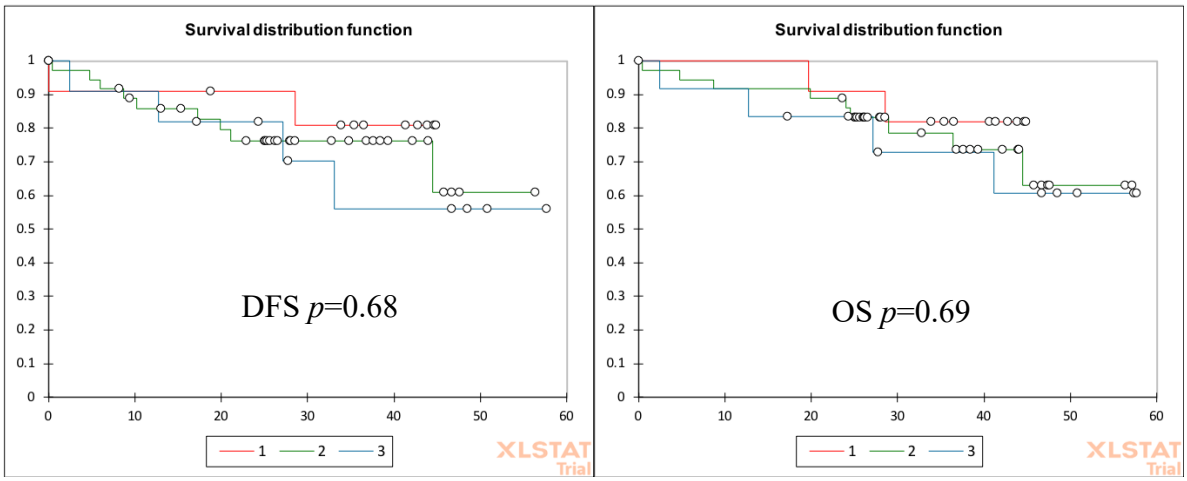
a. EMVI



b. Global MMP2 Cat Score.



c. Global TIMP2 Cat Score.



7.4.5 MMP2 Biomarker Test Optimisation

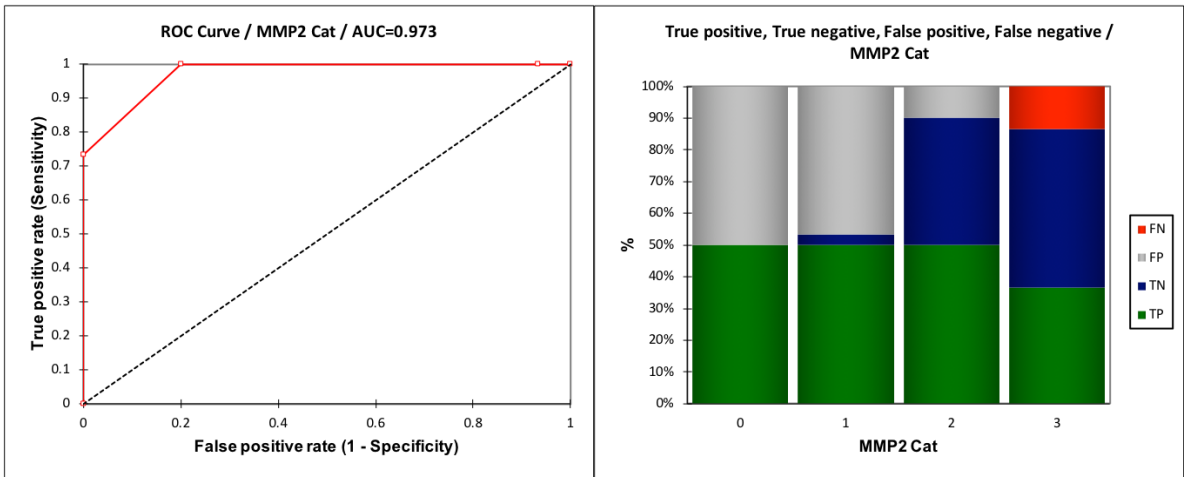
Global MMP2 Cat scores have been shown to be associated with EMVI on logistic regression analysis but the sensitivity and specificity thresholds for each Category has not been established. Additionally, sensitivity & specificity may be improved by test binarisation into MMP2-high or -low (combining Cat scores). **Table 7.2** demonstrates Primary and Secondary Binarisation strategies. Optimal binarisation is indicated by the threshold-based diagnostic accuracy analysis (table) and ROC curve in **Figure 7.5**, demonstrating superior sensitivity, specificity, and AOC for Primary Binarisation over Secondary Binarisation for global MMP2 Cat score (AOC 0.973 vs 0.947). These analyses indicate an optimal binarisation of individual Cat scores was produced by Primary Binarisation (accuracy 0.900).

Table 7.2 MMP2 Category Binarisation

MMP2	Primary Binarisation	Secondary Binarisation
Low	Cat 0 & Cat 1	Cat 0 to Cat 2
High	Cat 2 & Cat 3	Cat 3

Figure 7.5 MMP2 Test Breakdown and Binarisation Data

MMP2 Cat	Sensitivity	Specificity	PPV	NPV	TP	TN	FP	FN	Accuracy
0.000	1.000	0.000	0.500		30	0	30	0	0.500
1.000	1.000	0.067	0.517	1.000	30	2	28	0	0.533
2.000	**1.000**	**0.800**	**0.833**	**1.000**	**30**	**24**	**6**	**0**	**0.900**
3.000	0.733	1.000	1.000	0.789	22	30	0	8	0.867

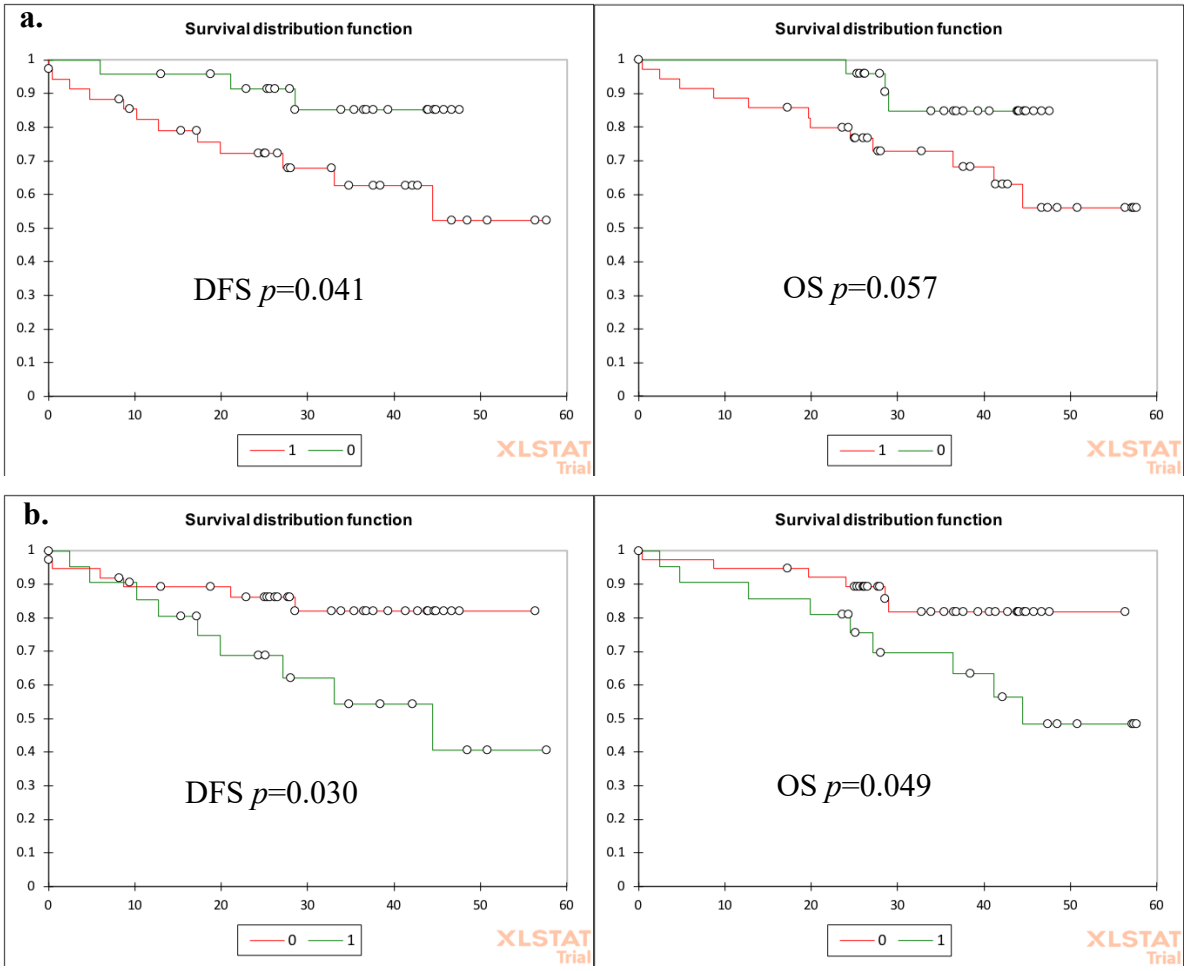


When binarisation (Primary or Secondary) is applied and Cox and KM modelling re-applied the hazard estimations indicate that Primary Binarisation is a better indicator of early adverse event than Secondary Binarisation, as demonstrated in **Table 7.3**. However, for DFS and OS based on KM analysis, Secondary Binarization of Cat scores was found to be significantly (p=0.030 & p=0.049) associated with MMP2 and outperformed Primary Binarisation (p=0.041 & p=0.057 [NS], respectively). These results are demonstrated in **Figure 7.6**.

Table 7.3 Cox Proportionate Hazard modelling for binarized MMP2 scores.

MMP2	Disease-free survival		Overall Survival	
	Hazard Ratio	*p*-value	Hazard Ratio	*p*-value
Primary Binarisation	3.45	0.033	3.21	0.047
Secondary Binarisation	2.97	0.037	-	0.055 [NS]

Figure 7.6 Kaplan-Mayer Survival Distribution Functions for Disease-Free (DFS) and Overall Survival (OS) assessed on **a.** Primary or **b.** Secondary binarized MMP2 scores.



7.4.6 CIMP, MMP2, & EMVI

When correlation between CIMP, MMP2 binarised score, and EMVI was examined, X^2-based contingency tables illustrated an association between CIMP and both MMP2 and EMVI in the sample population (p=0.004 and p<0.001, respectively). However, correlation matrix (Pearson) indicated a stronger and more significant association between EMVI positivity and MMP2 (Person=0.816, p<0.0001), than between CIMP and any other factor. These results are demonstrated in **Table 7.4** and **Appendix VI.d**.

Table 7.4 Pearson correlation matrix for CIMP, EMVI, and MMP2.

Variable	CIMP	EMVI	MMP2
CIMP	**1**	**0.415** p=0.001 CI 0.108 – 0.605	**0.367** p=0.004 CI 0.125 – 0.568
EMVI	**0.415** p=0.001 CI 0.108 – 0.605	**1**	**0.816** p<0.0001 CI 0.710 – 0.887
MMP2	**0.367** p=0.004 CI 0.125 – 0.568	**0.816** p<0.0001 CI 0.710 – 0.887	**1**

7.5 Discussion

The aims of this Chapter were to investigate any associations between the histopathological and clinical features of resected rectal tumours, metalloproteinase expression in these tumours, and CpG-methylator phenotype (CIMP); with a particular focus on EMVI. The data from this Chapter clearly demonstrate that, in this selected trial population, there was an association between tumours strongly expressing MMP2 and those demonstrating EMVI. This was demonstrated by global MMP2 scoring based on logistic regression modelling and the 4-teir system as described but was further improved by binarisation of the scoring system (AUC 0.947 vs 0.973). There was also an association between TIMP2 and EMVI, but TIMP2 was not a good discriminator between EMVI positive and negative tumours. No other marker demonstrated association with EMVI (MMP9 and TIMP4) and staining with MMP11 was unsuccessful.

Primary Binarised MMP2 scores demonstrated a sensitivity and specificity for EMVI positivity of 100% and 80%, respectively, with overall accuracy of 90% and a positive-predictive value of 88.3%. When this model of binarisation was tested against hazard (RR; Cox) and survival modelling (KM), relative risk for adverse DFS and OS was estimated at 3.45 and 3.21 in MMP2-positive tumours, respectively (both $p<0.05$), although this only translated into significant observed difference in DFS ($p=0.041$) based on Kaplan-Meyer analysis (OS was not significantly affected but was nearing significance ($p=0.057$)). Overall survival was however observed to be worse for those patients demonstrating higher MMP2 staining when a Secondary Binarisation protocol was employed, although the Cox hazard ratios were not as significant. Based on these assessments, the Primary Binarisation strategy produced the better accuracy when compared to Secondary Binarisation, produced significant RR and KM survival analysis, and was thus deemed to be a better test than Secondary Binarisation. Given the limited difference in significance for DFS and OS between the binarisation strategies, and the findings that insignificant *p* values were approaching threshold ($p<0.05$), there was only a minor degree of discrepancy between each of the two binarised scoring systems. However, care must be taken when indicating "trend", as this can often be misleading when assessing small populations[699].

When assessed as a factor associated with EMVI, MMP2 was more strongly associated with EMVI than CIMP, as demonstrated by superior Pearson correlation (0.816 vs 0.415), although both were significantly associated ($p<0.0001$ and $p=0.001$, respectively). This finding

correlates with the findings in **Chapter 3**; where although CIMP was associated with EMVI it did not translate into a predictor of survival. In the analysis in this Chapter, MMP2 Primary Binarised score was a predictor of EMVI and was associated with adverse survival. These findings have not however been validated in an unselected or external cohort, which is vital for the complete evaluation of a novel biomarker, although the findings do indicate its potential[700, 701].

Following the discovery of an association between Duke's stage and MMP2 abundance, the role of MMPs in colorectal cancer has been studied for some decades from both a mechanistic and biomarker persective[511]. Much of the recent interest in MMPs as biomarkers has fallen on circulating (serum) MMP9 and TIMP1, as these potential markers have been most powerfully associated with diagnosis. The quality of evidence for the association ranges from evidence derived from several small series where the identification and prognosis of colorectal cancers was found to correlate to circulating MMP9 activity (assessed by zymography) to much larger population based studies[702-704]. For instance, in a series of 748 asymptomatic patients, Wilson identified that serum MMP9 has a sensitivity and specificity of 79% and 70%, respectively, and thus might serve as a potential tool for rationalising screening colonoscopy when employed as part of a larger panel of genetic biomarkers[705]. However, more recent studies from the UK have indicated that MMP9 may be more useful as an adjunct to FOB testing in symptomatic patients entered into the screening programme, as the diagnostic accuracy is improved within the symptomatic population and augments faecal testing[679, 705]. A further study of 4509 symptomatic patients from Scandinavia indicated that serum TIMP1, in combination with CEA, was also a good diagnostic biomarker for colorectal cancers, but that its diagnostic accuracy was superior specifically for colonic tumours than for rectal tumours or the cohort as a whole[706]. When considering these studies, it must be noted that they are each examining serum levels of their respective biomarkers in either asymptomatic or symptomatic patient populations who then go on to have colonoscopy as a means of validating the test. This focus on diagnostic biomarkers has been driven by the prioritisation of preventative medicine and the high cost and resource burden associated with colonoscopy, especially given the high false-positive rates associated FOB and symptomatic screening, and thus the need for better discriminators to reduce "unnecessary" endoscopy[8, 328]. The patients in this study, by contrast, all have had rectal cancers resected with curative intent, and the test hypothesis was directed towards the detection of EMVI and thus is dissimilar to the screening population. However,

the investigation of staging and prognostic biomarkers in colorectal cancer, including the MMP family, is still a valid exploit as it may aid in delivering precision medicine[683, 707].

With regards prognosis, MMP9 and TIMP1 have previously shown some promise as potential biomarkers, and have been associated with worse DFS and OS, especially in compound metrics with pre-operative CEA[708, 709]. One study identified TIMP1 as a factor in predicting colorectal cancer liver metastasis and their recurrence, although VEGF was a better discriminator in this study[710]. The findings in this Chapter indicated that MMP2 but no other marker was associated with survival in terms of DFS and OS. This association between MMP2, EMVI, and survival is constructive, but the lack of association with MMP9 and TIMP2 is also important, especially since MMP9 has previously been found to be potentially useful as a screening tool or as a prognostic biomarker. Again, this study in comparison to the screening-oriented studies is highly selective, thus the results are unlikely to be representative of the whole population of colorectal cancers. However, this study's divergent findings may fit with the hypothesis that MSI-high hypermethylated tumours of the rectum form an under-recognised cohort of cancers with an atypical phenotype, including MMP expression, and a poorer prognosis.

Beyond survival data and metastasis, there has been some evidence to indicate that some MMPs may be valuable biomarkers in stratifying the response of cancers to chemotherapy, as has been found in gastric cancer[711, 712]. However, in colorectal cancer the data has been more inconsistent; two studies from Denmark, for instance, found that TIMP1 levels correlated to response in patients receiving combination irinotecan, 5-fluorouracil, and folinic acid, but not in patients receiving combination capecitabine and oxaliplatin, although baseline TIMP1 was a predictor of eventual progression-free survival[713, 714]. The findings in this study do not aid in predicting response to adjuvant therapy, although this would be an interesting avenue for investigation, as if MMP2 is a good predictor of EMVI and thus increase the number of patients considered for neoadjuvant therapy, knowing that those patients would have a significant response would valuable. Currently, only a limited number of studies have investigated MMP2 as a biomarker in predicting response to adjuvant chemotherapy. One found that an elevated ratio of MMP2 to MMP9 in osteosarcoma was associated with poor response, although another study in inflammatory breast cancer found exactly the opposite[715, 716].

Although associations between MMP2 staining, EMVI, and survival have been demonstrated in this chapter, and that MMP2 is a good predictor of EMVI, there are some limitations to its

application. Firstly, as already mentioned, the methodology employed in this Chapter relied upon the construction of TMAs form resected cancers, thus the ability to identify EMVI in these tumours is more than a little post hoc. For this method to be clinically useful it would need to be applied to biopsies taken at the point of diagnosis and initial staging so that if high MMP2 was identified, and therefore EMVI predicted, neoadjuvant therapies may be considered irrespective of MRI findings. Furthermore, as this trial population was highly selected, MMP2 as a predictor of EMVI would need to be validated against a large, unselected, external cohort.

7.6 Conclusions

In a selected trial population, MMP2 was a predictor of EMVI based upon the Primary Binarised score for staining. Higher MMP2 was also demonstrated to have a significant hazard ratio for DFS and OS and was significantly associated with poor DFS on Kaplan-Mayer analysis. OS for MMP2 did not quite reach significance. MMP2 score demonstrated better correlation to EMVI than did CIMP status. MMP2 has the potential to be useful as a diagnostic biomarker for EMVI in rectal cancers, and may also act as a prognostic biomarker, although further validation is required.

Chapter 8

General Discussion

8.1 Introduction

The overarching aim of this thesis was to investigate the relationship between methylation and EMVI in rectal cancers, its implications for prognosis, and seek to illuminate the biological processes that underlie any relationship. Three primary strands of enquiry were followed in order to achieve these aims; the investigation of the methylation status of resected rectal cancers and correlations to clinical outcomes; the *in vitro* manipulation of methylation to assess biologic effects on colorectal cell lines; and the investigation of putative epigenetic processes that may underlie the *in vitro* and *in vivo* observations. The investigation of potential biomarkers associated with EMVI was an additional objective, as it may serve to facilitate the staging and management of progressing rectal cancers. Each of these features is discussed in the context of consensus molecular subtyping in colorectal cancer and the potential for precision medicine[118, 201].

Throughout this Chapter the findings of this research are discussed and the overarching narrative pieced together. The limitations of each stage of the enquiry will be highlighted and complementary or tangential investigation suggested. The first comment in this vein, however, is the acknowledgement of the limitations of the principle investigator and author: As a clinician, stepping into the laboratory has been a challenging and rewarding process; methodologies and techniques that are the bread-and-butter of the undergraduate, graduate, and doctoral scientists have had to be learned from scratch and on-the-fly. The concepts of the scientific enquiry into cellular physiology and epi-/genetic investigation have all been novel and will inform my future clinical and academic insight, but as a novice there were hard barriers to overcome and thus the primary limitation in this investigation is my own limited expertise. On the other hand, my experience as a clinician and surgeon has greatly facilitated by insight into the translational aspects of this research and I hope that I have been able to support colleagues in the laboratory with clinical aspects of their own research, as they have supported me with the science.

8.2 The CpG Methylator Phenotype and Extramural Vascular Invasion

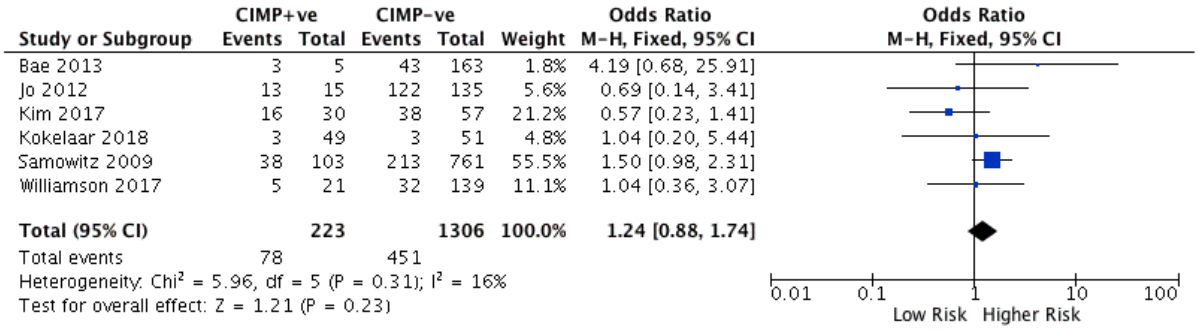
As highlighted in **Chapter 1** & **3**, the molecular and genetic aberrations that underlie CRC carcinogenesis are complex and not fully understood, although there is a consensus that there are divergent processes responsible for tumour development at different sites throughout the colon and rectum[118]. DNA methylation is one epigenetic process implicated in CRC, as well as other cancers, and CpG island methylator phenotype (CIMP) has drawn interest as a potential mechanism underlying both carcinogenesis and as a potential biomarker[148, 717]. CIMP, however, has primarily been associated with carcinogenesis in the right colon that is characterised by hypermethylation and microsatellite instability (MSI) (the serrated pathway), rather than the traditional chromosomal instability pattern typical of other sites in the colon and rectum[125, 136, 718]. Despite the preponderance in the right colon, CIMP tumours are known to occur in the rectum, although the clinical significance of this molecular tumour type occurring at this site is poorly understood, although some authors have suggested they represent a poor-prognostic subgroup[149, 719]. Many of the studies that have examined the role of methylation in rectal cancer or indeed colorectal cancer as a whole have focused on a single gene locus or have relied upon small cohorts, making outcomes interpretation challenging[353, 720].

The primary findings of **Chapter 3** were that a clear association was demonstrated between the presence of pEMVI and male sex, T and N stage, AJCC classification, and the requirement for adjuvant chemotherapy[258]. This study confirms the association of EMVI with worse clinicopathological features and that these translated into a statistically significant survival disadvantage, in keeping with the findings of other authors[280]. Although a significant relationship was demonstrated between CIMP-intermediate status and EMVI positivity (p=<0.001), this was not translated in to a disease-free or overall survival disadvantage for CIMP-intermediate patients (Log-rank p=0.66 and p=0.46, respectively). This was despite EMVI-positivity being an independent risk-factor for disease recurrence (Cox Proportional Hazard r=5.98 (1.10-32.50), p=0.038).

In order to gain a fuller understanding of the role of CIMP in EMVI in rectal cancer, a meta-analysis including the data gathered in **Chapter 3** was undertaken[721]. Briefly, a systematic search was performed of PubMed, Embase, MEDLINE, PubMed Central, and Cochrane electronic databases for articles pertaining to CIMP and rectal cancer. Articles were analysed and data extracted according to PRISMA standards – see **Appendix VI** for further detail. Six studies including 1529 patients were included in analysis[149, 204, 258, 318, 366, 719]. Following

dichotomisation, the prevalence of CIMP-positive tumours was 10% to 57%, with a median of 12.5%. Meta-analysis demonstrated the pooled odds ratio for all-cause death for CIMP-positive tumours vs CIMP-negative tumours was 1.24 (95% CI 0.88-1.74). Z-test for overall effect was 1.21 (p=0.23), therefore no significant association between CIMP and poor outcomes in rectal cancer was demonstrated (**Figure 8.1**). Heterogeneity between the studies was low (X^2 5.96, d.f. 5, p=0.31, I^2=16%) but total of 15 different loci were used for assessing CIMP across the studies, with a median of 6.5 loci (range 5-8). Another major finding was that rectal cancer datasets were frequently not extractable from larger colorectal cohorts, limiting analysis.

Figure 8.1 Pooled analysis on CIMP and overall survival (adapted from Kokelaar)[721].



From the investigation in **Chapter 3** and subsequent meta-analysis, there does not appear to be a significant relationship between CIMP and poor DFS or OS, despite the association between CIMP and EMVI. The differentiation in outcomes suggests an additional layer of complexity beyond a binary relationship between EMVI and CIMP. The significant limitation of all CIMP studies in all cancer types is the heterogeneity in assessing CIMP, a concern highlighted by many authors[148, 361]. With regards consensus molecular subtyping (CMS) of colorectal cancers, the original CMS study identified the frequency of CMS1 in the rectum as 3%, whereas this study of 100 consecutive patients with rectal cancers, given exclusions, demonstrated a higher than expected proportion of CIMP-intermediate and -high tumours (49%) which may be considered as analogous to CMS1 tumours. These findings must, however, be considered in the context of the methodology chosen to determine CIMP as other less discriminatory methods may have returned a lower frequency[185, 186]. The limited extractability of rectal datasets is also a constraint to analysis. Due to these limitations, the incidence of CIMP-high tumours in the rectum is probably under-reported.

8.3 Demethylating Agents in Colorectal Cell Lines

Chapter 4 investigated the biologic effects of demethylation on the colorectal cancer cell line DLD-1. The well characterised DNA-methyl transferase inhibitor azacytidine (AZA) was employed as the primary controlled variable to effect demethylation, but the putative demethylator RRx-001 was also investigated. These chemicals were first screen and optimised against standard tests for cytotoxicity and inhibition of cellular proliferation to ensure that the effects of biologic assays for invasion and migration were as a result of demethylation. Both agents demonstrated tolerable cytotoxicity at biologically effective doses. This was demonstrated by effective locus-specific and pan-DNA demethylation (msPCR and ELISA-like assays) at doses that were not cytotoxic but did reduce propensity of cells to migrate and invade.

The implications of the findings in this Chapter are twofold; firstly, they serve as a useful link to the findings in **Chapter 3**, as the same CIMP markers employed in assessing resected tumour methylation were employed, thus linking *in vivo* observations to potential *in vitro* processes. The second implication is that the findings serve to confirm the demethylating activity of the two investigated compounds and associate them to changes in cell behaviours (phenotype), thus allowing investigation of underlying mechanisms to be linked to both demethylation, cellular phenotype, and potentially tumour behaviour.

There were however limitations in each stage of this part of the investigation, although attempts at mitigation were made. With regards to the baseline investigation of cytotoxicity, only reasonably basic assays (RPD and trypan blue) were undertaken, although they were done in multiple biologic and technical replicates. Other more sophisticated methods of assessing cytotoxicity and the effects of potentially genotoxic agents are possible, although the methods employed in this study are well-established and accepted as adequate[439, 440, 722]. There was perhaps an additional element of concern in this assessment, however, when considering the investigation of RRx-001, which is not well characterised, and its modes of biologic action not well understood[235, 239]. The full investigation of new compounds in biologic processes, especially in humans with a view to potential therapeutic applications, is the basis of the pharmaceutical industry, and thus a more global and in-depth investigation of RRx-001 beyond the remit of this research. The addition of RRx-001 to the experimental construct was however insightful and will be pursued by the compound's proprietor, EpicentRx.

The assessment of DLD-1 cell propensity to migrate and invade based on scratch and Transwell assays was successful, although to some degree limited as biological models. The assessment by time-to-convergence was a simple and precise method of assessing cell activity and did not depend on more operator-dependent metrics that might have been more prone to confounding. Similarly, the Transwell system was effective at demonstrating the change in cell activity following demethylation and provided a link to EMT-like processes of early cancer progression due to the addition of an extracellular matrix component. These assays were, however, limited in their sophistication by the use of monoculture and monolayer techniques which do not model the tumour microenvironment well[447]. The addition of, for instance, tumour-associated macrophages or fibroblasts, could provide additional insight into the biologic activity of colorectal cancer cells in vitro and exposure to demethylating agents, as discussed in **Chapter 5** & **6** when considering EMT and metalloproteinase activity. Both scratch and Transwell systems are however widely used and accepted as robust methodologies for cellular investigation and the results gathered valid.

Finally, the demethylating effects of AZA and RRx-001 on DLD-1 cells was demonstrated by locus-specific and whole-DNA assays. These techniques showed that although both agents caused demethylation, it was to different degrees and at different rates; AZA causing a more profound demethylation at both levels, whereas RRx-001 demonstrating a slightly more dose-dependent whole-DNA effect and less effective locus-specific effect. The results for AZA are not unexpected as its mode of action is well characterised[216, 220]. Although identified as an alkylating agent, the demethylating action of RRx-001 is not understood, although it is apparently effective. However, the reduced ability of RRx-001 to demethylate at more locus-specific sites that AZA suggests that it's action as a demethylator is dependent on other genetic, epigenetic, or cellular events. One culprit in this effect may be chromatin restructuring, which has been shown to be important in regulating DNA methylation in metalloproteinases and other genes[592, 723].

8.4 Mechanisms Underlying the Response to Demethylation

Chapters 5 & **6** were designed to investigate the potential cellular mechanisms underlying the observed biologic effects of demethylation with AZA and RRx-001. The first stage in this process was identifying genes of interest that could then be investigated further, hence the initial approach was by a RT-PCR profiler array. As the array was only intended as a screening tool and to be controlled for demethylation, it was only performed once and only against AZA, as this was deemed to provide the best benchmarking for investigating the biologic effects. The RT^2 Profiler PCR Array – Human Tumour Metastasis (QIAGEN, Germany) was selected as it ties together the biologic effects observed *in vitro* (reduced migration and invasion) with the putative processes involved in EMVI *in vivo*. Arguably other arrays could have been chosen but the array selected provided coverage not only of genes associated with the EMT process, which is characterised by proliferative as well as early invasive changes, but also genes associated with metastasis. As the process of EMVI is not well understood as an individual biological process, the coverage of genes possibly associated locally invasive and locally metastatic processes is appropriate. Less focus was given to proliferative (primary tumour growth) and angiogenic (distantly metastatic) genes as they were deemed less likely to be important in the EMVI process. The array highlighted a number of up- and down-regulated genes, as well as many that did not change significantly, but the family of genes most that demonstrated the broadest and greatest magnitude in change was the metalloproteinases (MMPs) and their inhibitors (TIMPs).

As discussed in the respective Chapters, the MMPs and TIMPs are responsible for degrading and regulating the protein components of the ECM and have been implicated in a broad range of diseases, including colorectal cancer[683]. Due to the significant dysregulation demonstrated in the array, the preferred substrate of Type-IV collagen, and previous association with colorectal cancer drawn from the literature, the MMP2/TIMP2/MTP1-MMP axis was primarily investigated as a target for further expressional investigation by qRT-PCR. Complementary MMPs and TIMPs were also included for further depth of analysis. Following exposure to AZA, the qRT-PCR demonstrated a robust change in expression of MMP2, TIMP2, and TIMP4. Response in MMP11 and MMP13 was less pronounced and MMP9 uninterpretable due to reaction fouling. As MMP2 is the primary protease responsible for the degradation of collagen in the basement membrane, it's reduced expression in DLD-1 cells following demethylation may be causative in the reduced propensity of these cells to migrate and invade. The results obtained from RRX-001 were divergent from those of AZA, thus, due to this

inconsistency and previous discrepancy in demethylating effect, no further investigations were conducted with RRx-001.

The regulation and homeostasis of MMPs is complex, occurring at a transcriptomal, post-transcriptomal, and post-translational level. This was perhaps reflected in the finding that as well as the downregulation of MMP2, its primary inhibitor TIMP2 was also downregulated; challenging the notion that demethylation simply swings that balance of homeostasis towards anti-MMP2 activity. To discern between AZA having a direct transcriptional effect on MMP2 expression and one mediated via TIMP2 or TIMP4 in a homeostatic feedback mechanism, knockdown (KD) experiments and cross-correlation with PCR data was performed utilising proteomic techniques (immunocytochemistry/immunofluorescence and Western blotting). These experiments, however, proved challenging to interpret and suffered somewhat from fouling, although they did confirm the decrease of MMP2 protein abundance in response to AZA exposure. One limitation encountered in this phase of investigation was the inability to detect TIMP2 with ICC techniques, largely due to the protein predominantly being expressed extracellularly. This was despite other authors having success in detecting TIMP2 intracellularly under similar experimental conditions and the product literature suggesting it was possible. ICC was however successful for MMP2 and TIMP4 and corroborated the findings of PCR. Western blotting for MMP2, TIMP2, and TIMP4 was also undertaken, but this time was somewhat limited by a failure of the conjugation of the TIMP4 and secondary antibody in this protocol, the same primary antibody having probed adequately during ICC. Despite this there was sufficient data from WB to support the findings of the PCR for MMP2 and TIMP2, therefore providing proteomic cross-validation for each of the genes against PCR by one or other of the techniques.

When the proteomic data was considered in combination with the quantitative PCR, exposure to AZA was found to decrease MMP2 expression independently of TIMP2 and TIMP4, and thus could be assumed to be occurring via a direct effect of AZA on MMP2 transcription rather than via a homeostatic mechanism dependent on the expression of the counter-acting proteins. A greater understanding of the specific mechanism by which AZA prevents MMP2 expression could be addressed in further research, perhaps by focused study of the methylation of promotor sequences and transcriptomal binding sites by techniques such as methylation-specific pyrosequencing, but limited time and resources prevented this element (which would have required out-sourcing) being included in this study[724].

8.4 Metalloproteinases in Rectal Cancers & EMVI

Chapter 7 aimed to close the loop on the presence of EMVI in rectal cancers, the incidence of methylation assessed by CIMP-status, and the potential mechanisms underlying the relationship by examination of metalloproteinases and their inhibitors. The metalloproteinases were also investigated as potential biomarkers for the diagnosis of EMVI and as prognostic indicators. In the highly selected trial population, MMP2 was a predictor of EMVI based upon the Primary Binarised score. Higher MMP2 was also demonstrated to have a significant Cox hazard ratio for DFS and OS and was significantly associated with poor DFS on Kaplan-Mayer analysis. OS for MMP2 did not quite reach significance based upon Primary Binarisation but did on Secondary Binarisation. MMP2 score demonstrated better correlation to EMVI than CIMP status. These assessments were made on the basis of the scoring of immunohistochemically stained tissue microarrays where MMP2, MMP9, MMP11, TIMP2, and TIMP4 were targeted. MMP2 was the only factor identified that was a significant biomarker for diagnosis of EMVI and prognosis, although TIMP2 did demonstrate association with EMVI but was not a discriminator.

Although MMP2 has previously been associated with advancing Duke's stage in colorectal cancer, no previous study has identified MMP2 as a prognostic biomarker in rectal cancer, nor as a diagnostic biomarker in the identification of EMVI[511]. However, the method employed to assess MMP2 during this part of the research process is limited in that the trial population was highly selected, both in terms of the prevalence of EMVI (50% incidence of EMVI versus 44% in the unselected population in Chapter 3 and 17-70% in published series), and in the fact that all patients included in this research were those treated by surgery with curative intent and neoadjuvant naïve[271-273]. Its utility as a biomarker must therefore be tempered by this selectivity, and the need for large-scale validation in external cohorts. Furthermore, if MMP2 is to be considered as a viable biomarker, it would need to be examined in a test that can be applied at the time of diagnosis and staging such as blood test or IHC of biopsy specimens. Biopsies taken during the endoscopic diagnosis of rectal cancer are notorious for not being adequately diagnostic or representative of the tumour as a whole; partly due to tumour heterogeneity, but also due to the limitations of superficial sampling[477, 725, 726]. Serum detection of MMPs is feasible, but the specificity of MMP2 to rectal cancer (avoidance of confounding with comorbidities) and thresholds would need to be established and validated from scratch[683, 704].

8.5 Concluding Remarks

The overarching aim of this body of research was to investigate epigenetic events in rectal cancer and their relationship, if any, to EMVI. The initial hypothesis was that hypermethylation, as defined by the CpG island phenotype (CIMP), was associated with EMVI and this was demonstrated to be the case. However, CIMP status did not correspond to survival as EMVI does and does not in itself elucidate how methylation status may be mechanistically associated with EMVI. By use of demethylating agents, primarily 5-azacytidine (AZA), invasive colorectal cancer cells were rendered less invasive and at the same time dysregulation in metalloproteinase (MMP) expression was observed. The association between demethylation and MMP dysregulation was confirmed by means of knockdown and observation of biologic effect by both immunocytochemistry and Western blotting. As MMPs are vital in the epithelial-to-mesenchymal transition and to metastasis, due largely to their role in remodelling the extracellular matrix, they constitute a good mechanism for the observed *in vivo* effects of demethylation. This is particularly the case for MMP2, which is the primary protease responsible for degradation of type-IV collagen in the basement membrane. On assessment of resected tumours, MMP2 was found to be expressed significantly more strongly in tumours exhibiting EMVI those that did not, and MMP2 demonstrated good diagnostic accuracy in discriminating tumours with and without EMVI. MMP2 was also shown to be associated with survival and is a hazard for early poor outcomes in rectal cancer. By these means MMP2 may serve as a potential biomarker for the diagnosis of EMVI and in the prognosis of rectal cancers and serves as a means of understanding the fundamental biological processed underlying EMVI in rectal cancer.

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msPCR Primer Sequences & Reaction Conditions

Primer Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Annealing Temperature (°C)	Annealing Time (Secs)	Cycle number	Product Length (bp)
hMLH U	AGAGTGGARAGTGATTTTTAATGT	ACTCTATAAATTACTAAATCTCTTCA	52.5	35	37	93
hMLH M	GATAGCGATTTTTAACGC	TCTATAAATTACTAAATCTCTTCG	52.5	35	37	≈100
MINT1 U	AATTTTTTTATATATATTTTTGAAGTGT	AACAAAAAAACCTCAACCCCACA	55	35	37	≈100
MINT1 M	AATTTTTTTATATATATTTTCGAAGC	AAAAACCTCAACCCCGCG	55	35	37	100
SOCS1 U	TTTTTTGGTGTTGTTTGGAGGTTGGATTTT	AAAACAAAACAATAAACTAAAACACTACAAAACCA	50	35	37	243
SOCS1 M	TTGTTCGGAGGTCGGATTT	ACTAAAACGCTACGAAACCG	50	35	37	218
HAND1 U	AATAGTTTAGGGTGTTGGTT	AAATTTTACACTCAACCCA	55	30	35	184
HAND1 M	AATAGTTTAGGGCGTTGGTC	AATTTTACGCTCAACCCG	53	30	35	184
ADAMTS1 U	GTGAGTAATATTGTAGTTAAGGTGG	AAAACAAAAAACACTCTAAAACACC	58	30	35	101
ADAMTS1 M	GTGAGTAATATCGTAGTTAAGGCGG	CTAAAACAAAAAACGCTCTAAAACG	61	30	35	103
NEUROG1 U	TTGTTGGTTAATTGGTGGTGTTGT	CATTACCTCAACCACTAATCACCCA	59	30	35	119
NEUROG1 M	AATTTATGTTCGCGGGAGGTC	ACCAACTTAACCCGAACCGA	59	30	35	118
IGFBP3 U	TTATTTTGGTTTTTATATAGTGGTT	AACAAAAAACAACTAATCCTCAACA	51	35	35	≈100
IGFBP3 M	TTTCGGTTTTTATATAGCGGTC	AAAAAACGACTAATCCTCAACG	54	35	35	≈100
THBD U	ATGTGTTTGTTTTTATTTGGTGTT	CATAACTAACCAAAAACCCACA	57	30	35	158
THBD M	CGTTCGTTTTTATTCGGCGTC	GCCAAACCCCATCTCATCG	58	30	35	118

RTPCR Input Quality Control

NanoDrop RNA assessment

RNA-40	260	280	260/280	260/230	ng/uL
Blanks OK					
Control	7.468	3.503	2.13	1.50	298.7
vC1	8.168	3.849	2.12	0.98	326.7
vC2	6.736	3.171	2.12	0.68	269.4
vC3	6.989	3.275	2.13	0.98	279.6
1dA1	7.599	3.537	2.15	0.62	303.9
1dA2	6.604	3.107	2.13	1.16	264.2
1dA3	6.953	3.229	2.15	1.84	278.1
3dA1	3.326	1.529	2.18	0.29	133.0
3dA2	2.930	1.382	2.12	0.23	117.2
3dA3	3.273	1.538	2.13	1.88	130.9
3dR1	6.081	2.844	2.14	0.91	243.3
3dR2	6.304	2.955	2.13	1.34	252.2
3dR3	5.864	2.727	2.15	1.19	234.6
reblank ok					

NanoDrop cDNA quantification & normalisation

DNA-50	260	280	260/280	260/230	ng/uL
Blanks OK					
Control	35.536	18.986	1.87	2.20	1776.80
vC1	34.764	18.520	1.88	2.13	1738.20
vC2	35.521	18.988	1.97	2.07	1776.10
vC3	36.451	19.528	1.87	2.15	1822.60
1dA1(spot)	34.620	18.491	1.87	1.94	1731.00
1dA2	34.507	18.409	1.87	2.14	1725.40
1dA3	34.555	18.452	1.87	2.13	1727.70
3dA1	33.789	18.141	1.86	1.93	1689.50
3dA2	33.408	17.937	1.86	1.83	1670.40
3dA3	33.759	18.066	1.87	2.17	1687.90
3dR1(spot)	33.856	18.098	1.87	2.05	1692.80
3dR2	33.876	17.998	1.88	2.10	1693.80
3dR3	33.335	17.781	1.87	2.10	1666.80
reblank ok				AVE=	1723.00
				SD=	46.54857678
				AVE-1SD=	1676.45
				AVE+1SD=	1769.55

RTPCR Quantification Data

MMP2

	MMP2	bACT	Realtive to HK	Relative to Control
vCont	0.0000043306969642637	16290909.140280050	0.000000000000266	1.0000
1D AZA	0.0000030538663755296	11967462.348559990	0.000000000000255	0.9599
3D AZA	0.0000000844196074400	1455086.64121182	0.000000000000058	0.2182
3D RRX	0.0000063927427907786	5645616.89086577	0.000000000001132	4.2595

Biological Reps		MMP2	bACT	R HK	R Cont	SD	p	RtC
Control	a	0.00000596591439	24342375.09669230000000	2.45083E-13	1	0		
	b	0.00000428093991	11134428.19738370000000	3.84478E-13	1			
	c	0.00000274523659	13395924.12676540000000	2.04931E-13	1			
1D AZA	a	0.00000243912395	14361897.39122120000000	1.69833E-13	0.692959709	0.2798832	0.345806373	0.4950524
	c	0.00000120773531	11591997.30893720000000	1.04187E-13	0.297145027			
3D AZA	a	0.00000015178333	2492576.51933084000000	6.08941E-14	0.248462871	0.0131741	0.000074935	0.2391474
	b	0.00000003099262	1075052.19111463000000	2.88289E-14	0.229831886			
	c	0.00000007048288	797631.21319000100000	8.83652E-14	0.431195672			
3D RRX	a	0.00000568993205	5070907.68933563000000	1.12207E-12	4.578332276	0.2980132	0.001539356	4.7890594
	b	0.00001014961019	5279911.99031259000000	1.92231E-12	4.999786582			
	c	0.00000333868613	6586030.99294908000000	5.06934E-13	2.47368686			

		Relative Fold-Change	SD	p
	Control	1	0	
	24hrs 0.50uM AZA	0.495052368	0.279883246	0.346
	72hrs 0.50uM AZA	0.239147378	0.013174096	0.000
	72hrs 0.50uM RRx-001	4.789059429	0.298013198	0.002
		0.0000000000002782		

MMP11

	MMP11	bACT	Relative to HK	Relative to control
Control	0.0106527275	295.6546316445060	0.0000360309848	1.000
1D AZA	0.0182501810	251.9093844838590	0.0000724474040	2.011
3D AZA	0.0079434563	73.1002334957253	0.0001086652659	3.016
3D RRX	0.0091971004	178.6819160155820	0.0000514719151	1.429

Biological Reps

		MMP9	bACT	R HK	R Cont	SD	p	RtC
Control	a	0.0152422403525193009	358.29471055779300	4.25411E-05	1	0		1
	b	0.0092699118685296000	310.88595547259600	2.98177E-05	1			
	c	0.0074460304190928000	217.78322890312800	3.41901E-05	1			
1D AZA	a	0.0158803699645328000	298.46220015582800	5.32073E-05	1.250727996	1.09159706	0.0589375	2.252177
	b	0.0207598456956080000	203.82601684198800	0.000101851	3.415780864			
	c	0.0181103271976460000	253.43993645376000	7.14581E-05	2.09002207			
3D AZA	a	0.0096719170564383000	101.82634160668300	9.49844E-05	2.232770131	1.021140969	0.0092818	3.260019
	b	0.0060481875456200000	61.985656566220050	9.7574E-05	3.272348442			
	c	0.0081102643303700000	55.488702314272500	0.000146161	4.274940427			
3D RRX	a	0.0113141795484000000	206.05099175301300	5.49096E-05	1.290743505	0.31623488	0.0305193	1.471868
	b	0.0089062750185300000	162.59506398169700	5.47758E-05	1.837021512			
	c	0.0073708466404600000	167.39969231203600	4.40314E-05	1.287841293			

0.000035516298330

	RtC	SD
Control	1	0
24hrs 0.50uM AZA	2.252176977	1.09159706
72hrs 0.50uM AZA	3.260019667	1.021140969
72hrs 0.50uM RRx-001	1.47186877	0.31623488

MMP13

	MMP13	bACT	R to HK	R to Control
Control	0.013414229092501200000	33.404674791676700000000	0.000401567420612745000	1.00
1D AZA	0.006426855397582030000	27.785730642765700000000	0.000231300572232940000	0.58
3D AZA	0.004748805664909330000	15.193850483149000000000	0.000312547873903067000	0.78
3D RRX	0.010912583219594600000	25.577054294196500000000	0.000426655200167859000	1.06

Biological Reps

		MMP13	bACT	R HK	R Cont	SD		RtC
Control	a	0.01781333265517	38.28571718392540	0.000465274	1	0		1
	b	0.01052692850921	30.70886416442970	0.000342798	1			
	c	0.01190242611312	31.21944302667490	0.00038125	1			
24hrs 0.50uM AZA	a	0.00633897503081	32.26437866895140	0.00019647	0.42226 7195	0.03299 6996	0.024 1661	0.701 3985
	b	0.00657188287138	26.45301255300320	0.000248436	0.72473 0863			
	c	0.00636970829055	24.63980070634230	0.000258513	0.67806 6064			
72hrs 0.50uM AZA	a	0.00545618696941	17.01189928784530	0.000320728	0.68933 1381	0.11178 8527	0.306 5912	0.768 3778
	b	0.00443191229175	15.07978519672670	0.000293898	0.00034 2798			
	c	0.00435831773357	13.48986696487510	0.000323081	0.84742 4231			
72hrs 0.50uM RRx-001	a	0.01179629192676	24.87385208615840	0.000474245	1.01928 1338	0.42179 519	0.125 3035	1.317 5356
	b	0.01432146426390	25.85619634120390	0.000553889	1.61578 9816			
	c	0.00661999346813	26.00111445522730	0.000254604	0.66781 3593			

		RtC	0.000396440575479 SD
	Control	1	0
	24hrs 0.50uM AZA	0.701398464	0.032996996
	72hrs 0.50uM AZA	0.768377806	0.111788527
	72hrs 0.50uM RRx-001	1.317535577	0.42179519

TIMP2

	TIMP2	bACT	Relative to HK	Relative to Control
Control	0.2411600769635	3.8201304675078	0.0631287541132	1.0000
1D AZA	0.2678045305019	3.8491762630156	0.0695745043102	1.1021
3D AZA	0.0395042563544	1.7578163407273	0.0224734834005	0.3560
3D RRX	0.2298000344371	3.0603892303182	0.0750884992538	1.1895

Biological Reps

		TIMP2	bACT	R HK	R Cont	SD	p	RtC
Control	a	0.33204094096041	4.72275064671254	0.070306685	1	0		1
	b	0.21108259988091	3.63587757278801	0.058055475	1			
	c	0.18035669004905	3.10176318302270	0.058146506	1			
1D AZA	a	0.29722828860285	4.66067602879405	0.063773643	0.070306685	0.6986845	0.382801675	0.8712702
	b	0.25650767421782	3.71891332274507	0.068973824	1.18806752			
	c	0.24967762868495	3.16793943750778	0.078813889	1.355436389			
3D AZA	a	0.04354987832252	2.02772660910160	0.021477194	0.305478694	0.0603865	0.00002720	0.3678148
	b	0.03694993526837	1.71126184093322	0.021592216	0.371923853			
	c	0.03801295547243	1.53446057214706	0.024772846	0.426041871			
3D RRX	a	0.23524654348059	3.08568767016791	0.076237963	1.084362939	0.3141303	0.139453856	1.2269977
	b	0.26548180295319	2.88122099527059	0.092142117	1.587139149			
	c	0.18867175687737	3.21425902551607	0.058698367	1.009490885			

		RtC	0.062169555129067 SD
	Control	1	0
	24hrs 0.50uM AZA	0.871270198	0.698684476
	72hrs 0.50uM AZA	0.367814806	0.060386531
	72hrs 0.50uM RRx-001	1.226997657	0.314130349

TIMP4

	TIMP4	bACT	Relative to HK	Relative to Control
Control	0.00113	9573.92110	1.17886E-07	1
1D AZA	0.00148	6261.27349	2.35669E-07	1.999128973
3D AZA	0.00044	1124.13516	3.92426E-07	3.328864832
3D RRX	0.00064	3806.41091	1.6823E-07	1.427054421

1

1

1

Biological Reps

		TIMP4	bACT	R HK	R Cont	SD	p	RtC
Control	a	0.00178	17433.32019	1.02373E-07	1	0		1
	b	0.00024	5314.60594	4.5459E-08	1			
	c	0.00136	5973.83717	2.27591E-07	1			
1D AZA	a	0.00132	7014.75685	1.8787E-07	1.835151175	0.55058597	0.20109893	1.4458281
	c	0.00155	6425.54087	2.40451E-07	1.056505028			
3D AZA	a	0.00054	1147.00617	4.71281E-07	4.603564461	1.8140859	0.0312613 25	5.8863169
	b	0.00040	1237.77796	3.25899E-07	7.169069348			
	c	0.00038	987.62135	3.84224E-07	1.688220334			
3D RRX	a	0.00103	4087.06570	2.53144E-07	2.472763084	0.65461017	0.0263526 22	2.9356423 7
	b	0.00051	3290.86637	1.54494E-07	3.398521665			

1.25141E-07

	RtC	SD
Control	1	0
24hrs 0.50uM AZA	1.445828101	0.550585971
72hrs 0.50uM AZA	5.886316904	1.814085903
72hrs 0.50uM RRx-001	2.935642374	0.65461017

Proteomics Raw Data

Western Blot (Normalised) Data

Prot Fold Change	MMP2	TIMP2	TIMP4
Control 1	1	1	x
Control 2	1	1	x
Control 3	1	1	x
AZA 1	0.26	0.29	x
AZA 2	0.47	0.39	x
AZA 3	0.32	0.4	x
KD T2 1	0.94	0.02	x
KD T2 2	1.1	0.05	x
KD T2 3	1.04	0.12	x
KD T4 1	1.07	0.92	x
KD T4 2	0.86	0.99	x
KD T4 3	1.09	1.06	x
KD T2&4 1	1.12	0.05	x
KD T2&4 2	0.89	0.1	x
KD T2&4 3	0.97	0.08	x
KD T2 + AZA 1	0.32	0.1	x
KD T2 + AZA 2	0.27	0.02	x
KD T2 + AZA 3	0.24	0.06	x
KD T4 + AZA 1	0.33	0.24	x
KD T4 + AZA 2	0.41	0.33	x
KD T4 + AZA 3	0.29	0.4	x
KD T2&4 + AZA 1	0.41	0.03	x
KD T2&4 + AZA 2	0.37	0.05	x
KD T2&4 + AZA 3	0.26	0.12	x

	MMP2	TIMP2	TIMP4
Control	1	1	x
AZA	0.35	0.36	x
KD T2	1.026666667	0.063333333	x
KD T4	1.006666667	0.99	x
KD T2&4	0.993333333	0.076666667	x
KD T2 + AZA	0.276666667	0.06	x
KD T4 + AZA	0.343333333	0.323333333	x
KD T2&4 + AZA	0.346666667	0.066666667	x

	MMP2	TIMP2
Control SD	0	0
AZA SD	0.108166538	0.060827625
KD T2 SD	0.080829038	0.051316014
KD T4 SD	0.127410099	0.07
KD T2&4 SD	0.116761866	0.025166115
KD T2 + AZA SD	0.040414519	0.04
KD T4 + AZA SD	0.061101009	0.080208063
KD T2&4 + AZA SD	0.077674535	0.047258156

	MMP2	TIMP2	M vs T SdT
Control SdT	-	-	
AZA SdT	0.009	0.003	0.852
KD T2 SdT	0.625	0.001	0.002
KD T4 SdT	0.936	0.828	0.856
KD T2&4 SdT	0.930	0.000	0.008
KD T2 + AZA SdT	0.001	0.001	0.009
KD T4 + AZA SdT	0.003	0.005	0.788
KD T2&4 + AZA SdT	0.005	0.001	0.060

Immunocytochemistry RapidScore (Normalised) Data

RapidScore	MMP2	TIMP2	TIMP4
Control 1	1	1	1
Control 2	1	1	1
Control 3	1	1	1
AZA 1	0.25	x	4.21
AZA 2	0.43	x	3.2
AZA 3	0.35	x	3.8
KD T2 1	0.99	x	1.01
KD T2 2	1.09	x	1.03
KD T2 3	1.15	x	0.99
KD T4 1	1.12	x	1.01
KD T4 2	0.86	x	1.05
KD T4 3	0.92	x	1.02
KD T2&4 1	1.2	x	1.03
KD T2&4 2	0.91	x	1.01
KD T2&4 3	0.99	x	0.98
KD T2 + AZA 1	0.32	x	3.67
KD T2 + AZA 2	0.24	x	5.72
KD T2 + AZA 3	0.42	x	2.9
KD T4 + AZA 1	0.33	x	1.01
KD T4 + AZA 2	0.47	x	0.98
KD T4 + AZA 3	0.25	x	1.02
KD T2&4 + AZA 1	0.41	x	1.08
KD T2&4 + AZA 2	0.37	x	1.04
KD T2&4 + AZA 3	0.26	x	0.99

	MMP2	TIMP2	TIMP4
Control	1	1	1
AZA	0.343333333	1	3.736666667
KD T2	1.076666667	1	1.01
KD T4	0.966666667	1	1.026666667
KD T2&4	1.033333333	1	1.006666667
KD T2 + AZA	0.326666667	1	4.096666667
KD T4 + AZA	0.35	1	1.003333333
KD T2&4 + AZA	0.346666667	1	1.036666667

	MMP2		TIMP4
Control SD	0	0	0
AZA SD	0.090184995	0	0.507969815
KD T2 SD	0.080829038	0	0.02
KD T4 SD	0.136137186	0	0.02081666
KD T2&4 SD	0.149777613	0	0.025166115
KD T2 + AZA SD	0.090184995	0	1.457612203
KD T4 + AZA SD	0.111355287	0	0.02081666
KD T2&4 + AZA SD	0.077674535	0	0.045092498

	MMP2	TIMP4	M vs T SdT
Control SdT	-	-	
AZA SdT	0.006	0.011	0.010
KD T2 SdT	0.242	0.478	0.329
KD T4 SdT	0.713	0.157	0.569
KD T2&4 SdT	0.737	0.691	0.766
KD T2 + AZA SdT	0.006	0.067	0.052
KD T4 + AZA SdT	0.010	0.808	0.013
KD T2&4 + AZA SdT	0.005	0.294	0.001

Immunohistochemistry Scoring Sheet

Scoring system for IHC
(with reference to Fedchenko and Reifenrath, 2014)

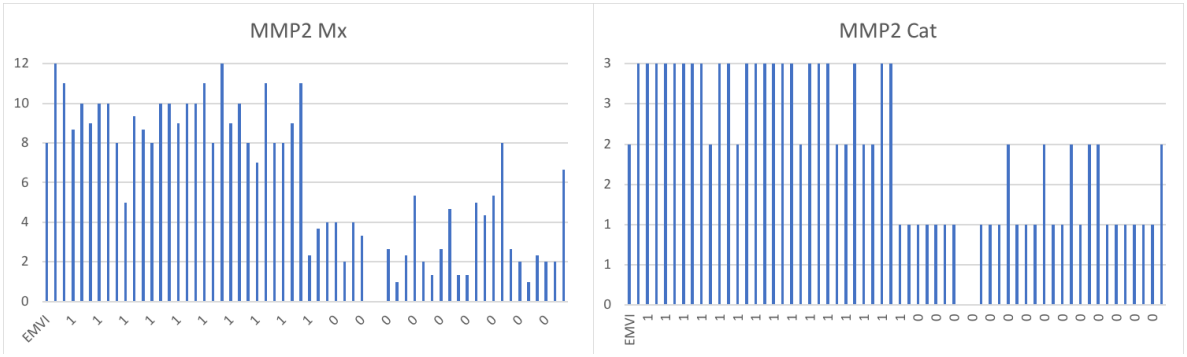
Proportion of Cells	p-Score	Intensity of Staining	i-Score
None	0	**None**	0
<10%	1	**Weak**	1
10% – 50%	2	**Intermediate**	2
51% - 90%	3	**Strong**	3
>90%	4		
		MAX SCORE	**7**

MMP2 MMP9 MMP11 TIMP2 TIMP4

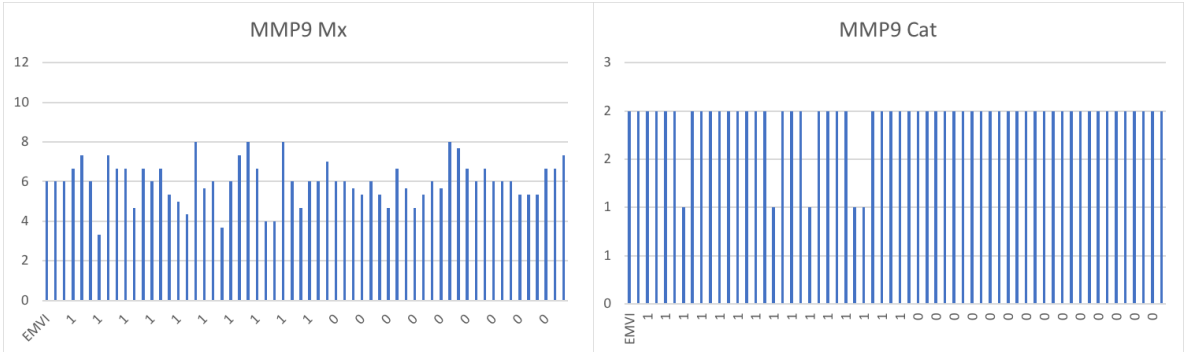
Path Number	**p-score**	**i-score**	**p-score**	**i-score**	**p-score**	**i-score**
Rep…	**A**		**B**		**C**	
SH1015214						
MH127050						
SH1001950						
SH1003461						
SH1014485						
SH1016041						
SH1204044						
SH1115961						
SH1301209						
SH1125646						
SH105224						
SH1101201						
SH1242267						
SH1006849						
SH1211596						
MH1100593						
SH1300417						
SH1005494						
SH1014844						
MH1204971						
SH1209984						
etc…						
etc…						

Frequency histograms demonstrating multiplied metric (Mx) and Categorised staining scores for **a.** MMP2; **b.** MMP9; **c.** TIMP2; and **d.** TIMP4.

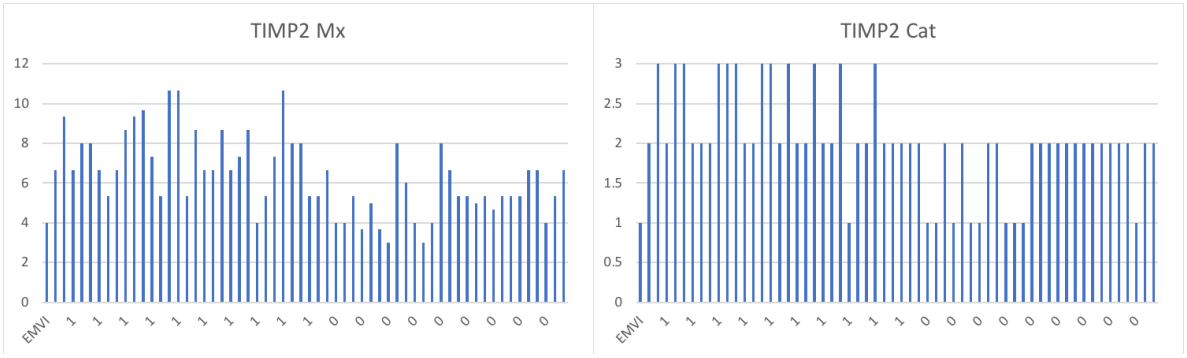
a.



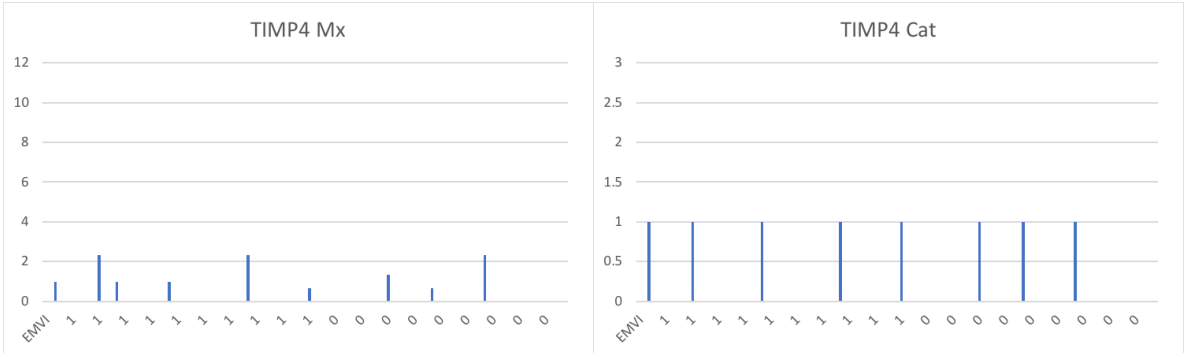
b.



c.



d.

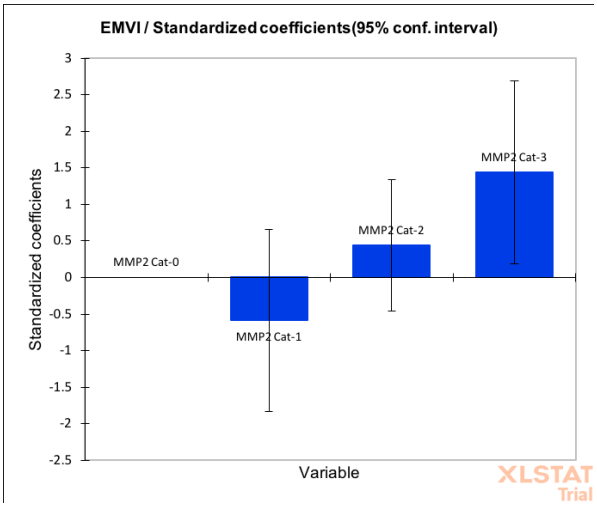


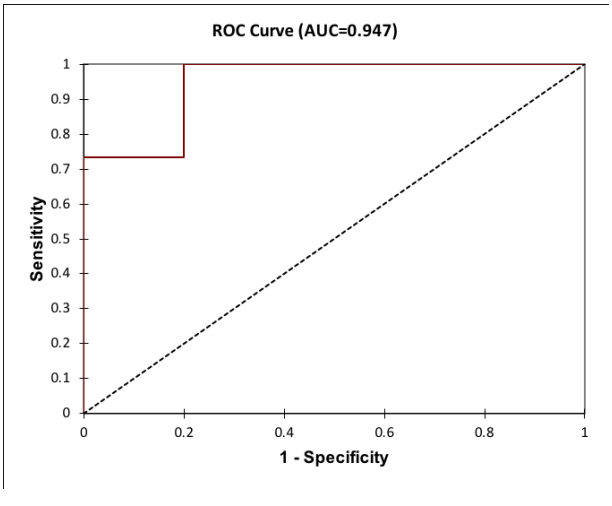
Logistic regression modelling for EMVI in relation to nominal categorised (Cat) scores for **a.** MMP2 and **b.** TIMP2

a. MMP2 Summary Statistics & Test of the Null Hypothesis H0: Y=0.5.

Variable	**Categories**	**Frequencies**	**Percentage**		**Statistic**	**DF**	X^2	**PR>X^2**
EMVI	1	30	50%		-2 Log	3	64.467	< 0.0001
	0	30	50%		Score	3	46.286	< 0.0001
					Wald	3	14.477	0.002
Cat Score	0	2	3.3%					
	1	22	36.7%					
	2	14	23%					
	3	22	36.7%					

Standardised Coefficient Model & ROC Curve.





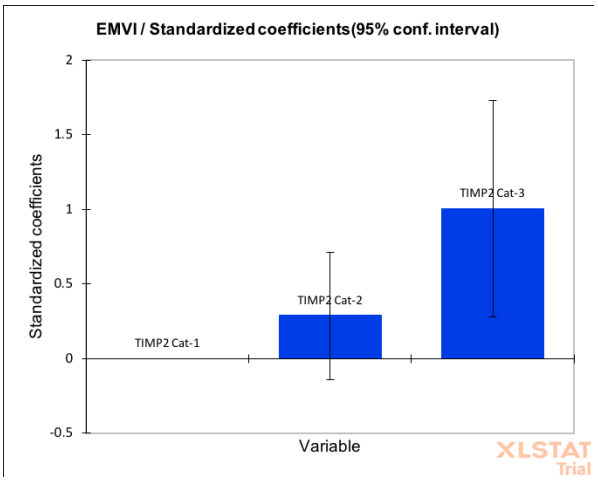
Classification Table for the training sample.

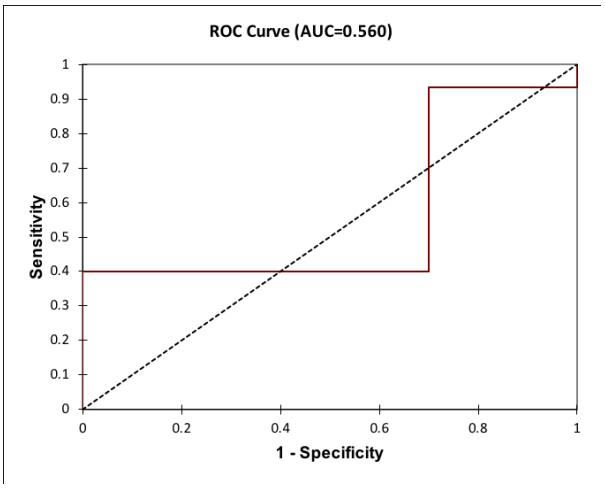
from / to	**0**	**1**	**Total**	**% correct**
0	24	6	30	80.0%
1	0	30	30	100.0%
Total	24	36	60	90.0%

b. TIMP2 Summary Statistics & Test of the Null Hypothesis H0: Y=0.5.

Variable	Categories	Frequencies	Percentage		Statistic	DF	X^2	PR>X^2
EMVI	1	30	50%		-2 Log	2	26.406	< 0.0001
	0	30	50%		Score	2	17.130	0.0002
					Wald	2	7.450	0.024
Cat Score	1	11	18.3%					
	2	37	61.7%					
	3	12	20.0%					

Standardised Coefficient Model & ROC Curve.





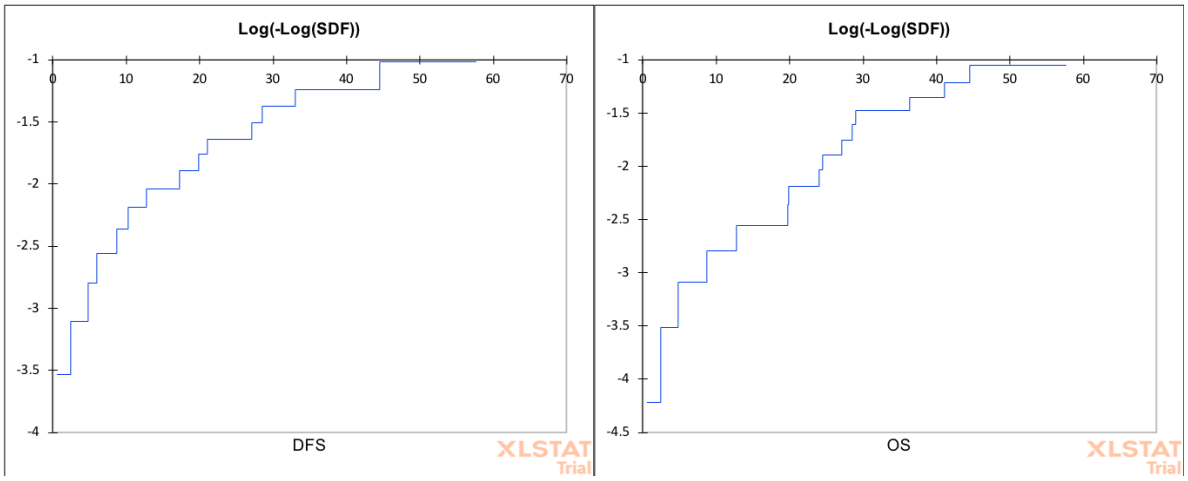
Classification Table for the training sample.

from / to	**0**	**1**	**Total**	**% correct**
0	30	0	30	100.0%
1	18	12	30	40.0%
Total	48	12	60	70.0%

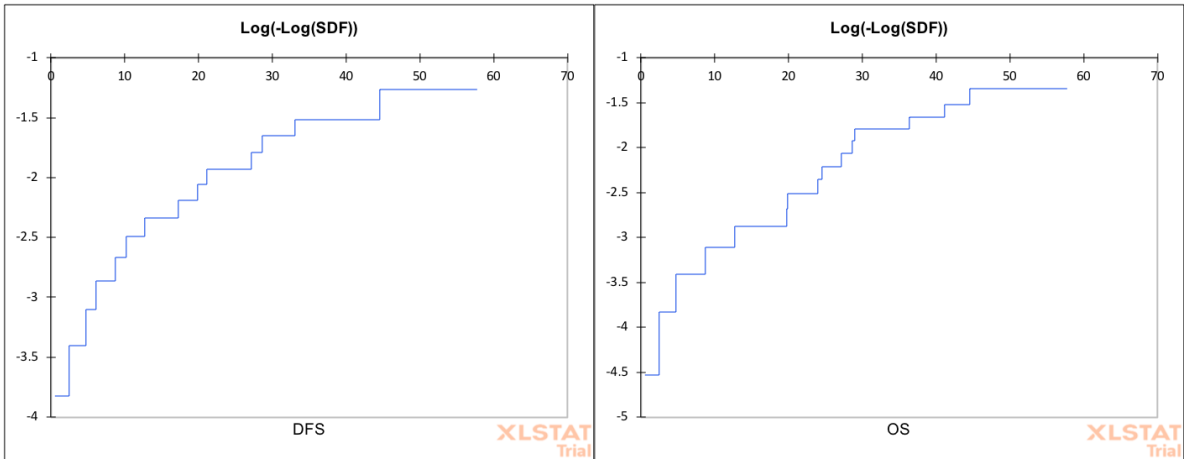
Cox Proportionate Hazard modelling for Disease-Free (DFS) and Overall Survival (OS) assessed on EMVI and categorised staining (Cat) scores for MMP2 & TIMP2.

(HR, Hazard Ratio; T, Statistical Trend; NS, Statistical Non-Significance)

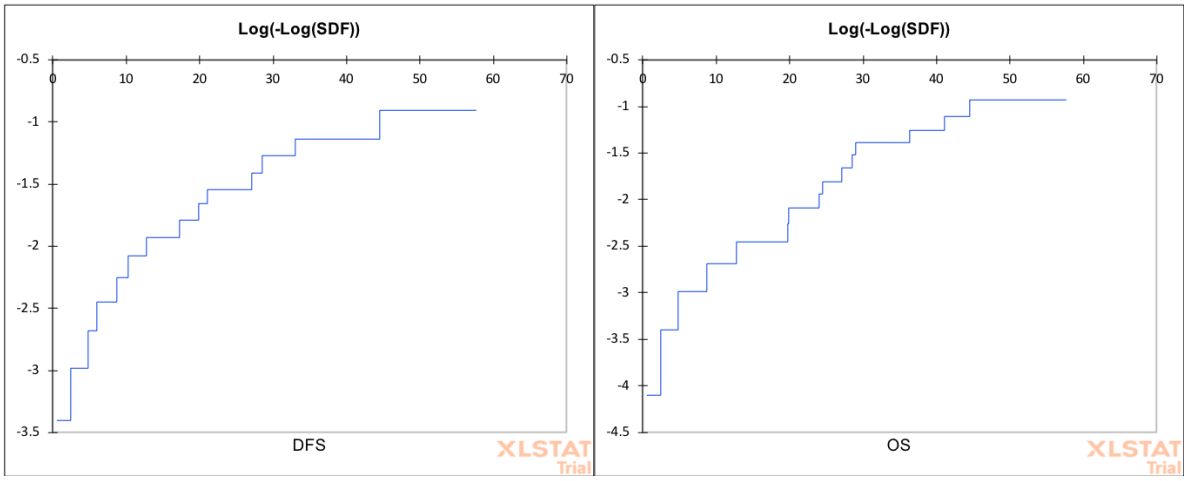
a. EMVI. (DFS HR 3.32, *p*=0.027; OS HR 3.05, *p*=0.043)



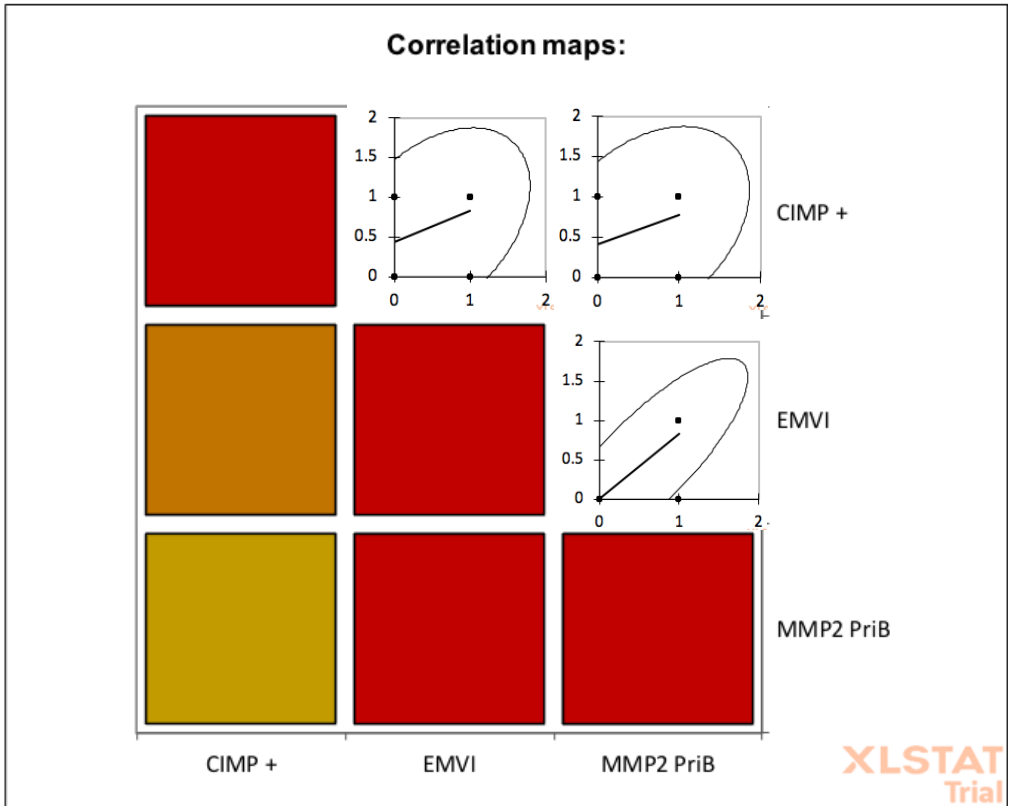
b. MMP2. (DFS *p*=0.11, OS *p*=0.15)



c. TIMP2. (DFS *p*=0.66, OS *p*=0.67)



Heat-Map and Scatter-Plots of Pearson's Correlations between CIMP, EMVI, and MMP2.



Meta-analysis Search Strategy & Consort Diagram

Aim: Meta-analysis of CIMP in rectal tumours and relationship to outcomes (DFS/OS).

Boolean Search Term: CpG island methylator phenotype AND (cancer OR carcinoma OR adenocarcinoma OR tumor OR tumour) AND (colorectal OR rectal) AND (prognosis OR outcome)

PubMed: 223

Cochrane: 8

Medline: 10

After duplications removed: 203

Excluded based on title/abstract: 160

Not colorectal cancer - 2

Non-CpG methylation focus of analysis – 89

Single gene methylation only – 3

Not related to prognosis – 59

Review articles - 7

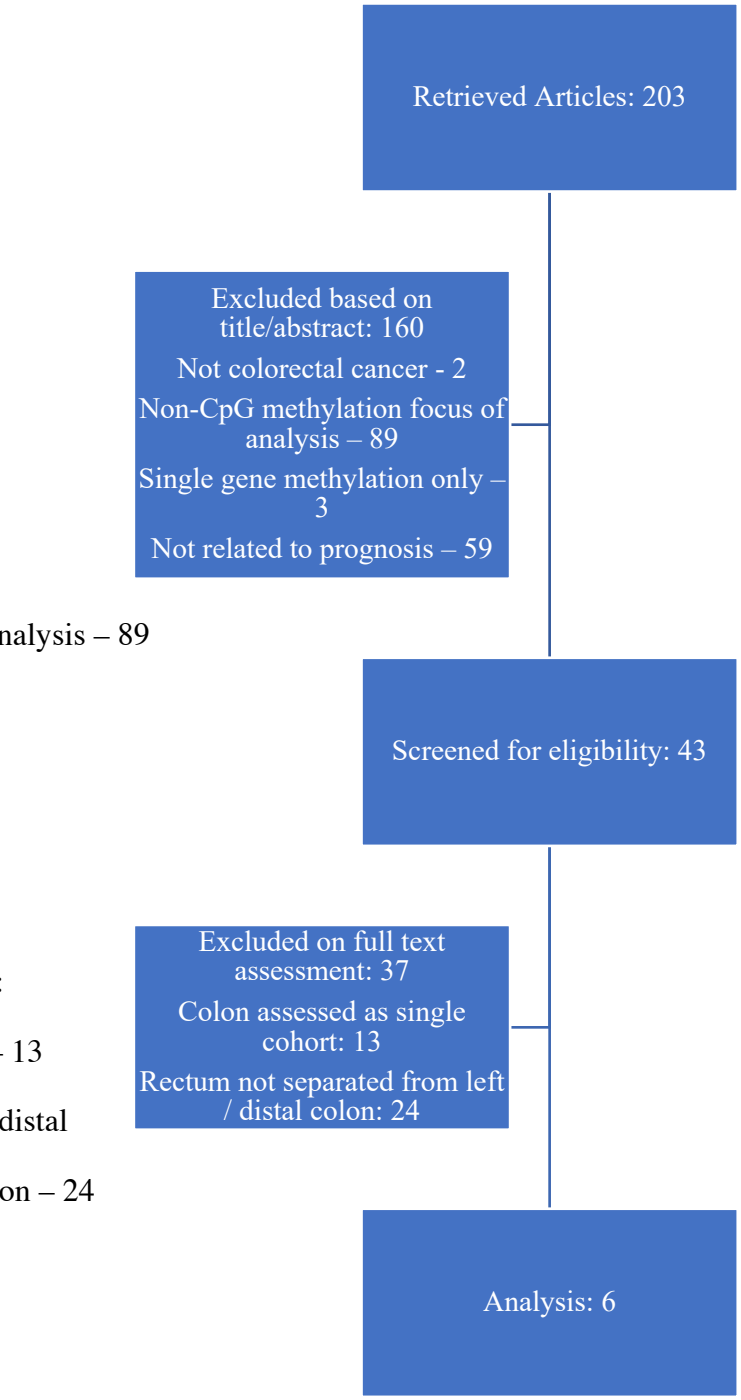
Screened for eligibility: 43

Excluded on full text assessment:

Colon assessed as single cohort – 13

Rectum not separated from left / distal colon – 24

Analysis: 6



Characteristics of Included Studies

Reference	**# Patients**	**Study Interval**	**Age Range (Mean)**	**Men (%)**	**AJCC**	**nCRT**	**N-O Score**
Samowitz *et.al.* 2009	864	1997-2001	30-79 (nr)	nr	I-IV	nr	6
Jo *et.al.* 2011	150	2004-2006	nr (61)	71	II-IV	NO	6
Bae *et.al.* 2013	168	2004-2006	36-87 (62)	67	I-IV	NO	7
Williamson *et.al.* 2017	160	2002-2011	nr (65)	71	II-IV	YES	7
Kim *et.al.* 2017	87	2006-2007	31-88 (65)	59	I-IV	nr	7
Kokelaar *et.al. 2018*	100	2010-2013	24-89 (71)	70	I-IV	NO	7