Analysis of Novel Cellular Responses and Serological Markers in Colorectal Cancer Patients

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Abstract

In recent years there have been significant advances in the treatment of cancer. This has been particularly aided by the discovery and exploitation of checkpoint inhibition; immunotherapy has the potential to induce sustained responses and increased survival in some patients. In solid tumours such as colorectal cancer (CRC), however, these therapies only benefit a small subgroup of patients. This is thought to be a result of poor tumour immunogenicity and low immune cell infiltration. In light of this, efforts are underway to identify new immunotherapeutic approaches to both alleviate tumour-mediated immunosuppression, and to increase anti-tumour immune responses.

The Godkin/Gallimore research group has recently completed a clinical trial, “TroVax and Cyclophosphamide Treatment in Colorectal Cancer” (TaCTiCC), in which the ability of cyclophosphamide (CPM) and 5T4 cancer vaccine TroVax to induce immune responses in advanced metastatic CRC (mCRC) patients was assessed. Unexpectedly, these treatments were found to not only induce immune responses but were also associated with increased survival in a proportion of patients, therefore may represent new treatment options for mCRC patients. Given the rapid explosion in the number of available immunotherapies, it is vital that we understand which patients will benefit from treatment. This thesis investigates a panel of plasma proteins and serological/immunological markers to identify mCRC patients who responded to CPM and TroVax on the TaCTiCC trial; several potential biomarkers of response to both therapies have been identified. Plasma proteins were also assessed for their ability to identify earlier-stage and mCRC patients both from each other, and from healthy donors; there is a clear and significant difference in circulating plasma proteins between these groups. Finally, this thesis investigates T cell responses to 7 novel CRC tumour antigens recently identified within the Godkin/Gallimore group; responses to several antigens are reduced in CRC patients compared to healthy donors and may therefore represent useful immunotherapy targets.

It is hoped that the findings described in this thesis will contribute to the improved identification of CRC, and to our understanding of factors associated with immunotherapy response. Moreover, the identification of novel CRC antigen targets may inform the development of improved immunotherapies.

List of Abbreviations

3,3'-diaminobenzidine	DAB
4-1BB ligand	4-1BBL
Adenomatous polyposis coli	APC
Alanine aminotransferase	ALT
Antibody	Ab
Antigen presenting cell	APC
Apolipoprotein A1	APOA1
Area under curve	AUC
Arylsulfatase family member J	ARSJ
B cell receptor	BCR
B- and T-lymphocyte attenuator	BTLA
Bacillus Calmette-Guérin	BCG
Brain-derived neurotrophic factor	BDNF
C-reactive protein	CRP
Cancer antigen 19-9	CA 19-9
Cancer stem cell	CSC
Carcinoembryonic antigen	CEA
Carcinoembryonic antigen related cell adhesion molecule 3	CEACAM3
Centromere protein Q	CENPQ

Chemokine ligand 17	CCL17
Chemokine receptor 4	CCR4
Chimeric antigen receptor	CAR
Cluster of differentiation	CD
Colorectal cancer	CRC
Colorectal cancer subtyping consortium	CRCSC
Combination of fluorouracil (5FU), leucovorin, and oxaliplatin	FOLFOX
Computed tomography	CT
Consensus molecular subtypes	CMS
CpG island methylator phenotype	CIMP
Cyclin-dependent kinase 4	CDK4
Cyclin-dependent kinase 8	CDK8
Cyclophosphamide/methotrexate/5-fluorouracil	CMF
Cytochrome P450 Family 2 Subfamily B Member 6	CYP2B6
Cytotoxic T-lymphocyte-associated protein 4	CTLA-4
Dendritic cell	DC
Distyrene, a plasticizer, and xylene	DPX
DNAJ heat shock protein family (Hsp40) member B7	DNAJB7
Epidermal growth factor receptor	EGFR
Epithelial cell adhesion molecule	EpCam

Epithelial-to-mesenchymal transition	EMT
Extracellular matrix	ECM
Faecal immunohistochemistry test	FIT
Familial adenomatous polyposis	FAP
Fetal calf serum	FCS
Forkhead transcription factor 3	Foxp3
Full blood count	FBC
Gastrointestinal	GI
Haemagglutinin	HA
Haematocrit	Haem
Haemoglobin	Hb/Hgb
Heat shock protein 40kD	Hsp40
Heparin-binding epidermal growth factor precursor	proHB-EGF
Hepatitis B virus	HBV
Hepatocellular carcinoma	HCC
Hepatocyte growth factor	HGF
Human epidermal growth factor receptor 2	HER2/neu
Human papillomavirus	HPV
Immune dysregulation, polyendocrinopathy, enteropathy, X-linked	IPEX
Immunohistochemistry	IHC
Indoleamine 2,3-dioxygenase	IDO

Inducible costimulator	ICOS
Inflammatory bowel disease	IBD
Innate lymphoid cell	ILC
Interferon	IFN
Interferon gamma-induced protein-10 (also known as CXCL10)	IP-10
Interleukin	IL
Lymphocyte-activating gene 3	LAG-3
Lysyl oxidase	LOX
Magnetic resonance imaging	MRI
Major histocompatibility complex	MHC
Matrix metalloproteinase	MMP
Mean corpuscular haemoglobin	MCH
Mean corpuscular volume	MCV
Melanoma antigen recognised by T cells 1	MART-1
Melanoma-associated antigen 1	MAGEA1
Metastatic colorectal cancer	mCRC
Methylcholanthrene	MCA
Microsatellite instability	MSI
Microsatellite instability high	MSI-H
Microsatellite-stable	MSS
Mismatch repair	MMR

Mismatch-repair-deficient	dMMR
Mismatch-repair-proficient	pMMR
Mucin 1	MUC1
Myeloid-derived suppressor cell	MDSC
National Institute for Health and Care Excellence	NICE
Natural killer cell	NK cell
Natural killer T cell	NKT cell
Neutrophil extracellular traps	NETs
Neutrophil:Lymphocyte ratio	NLR
New York oesophageal squamous cell carcinoma	NY-ESO-1
Non-small-cell lung cancer	NSCLC
Pathogen recognition receptor	PRR
Pathogen-associated molecular pattern	PAMP
Peripheral blood mononuclear cell	PBMC
Phycoerythrin	PE
Phytohemagglutinin	PHA
Placental growth factor 1	PIGF-1
Programmed cell death protein 1	PD-1
Programmed death-ligand 1	PD-L1
Prostate-specific antigen	PSA
Purified protein derivative of tuberculin	PPD

Reactive oxygen species	ROS
Receiver-operating characteristic	ROC
Recombination-activating gene 2	RAG-2
Red blood cell	RBC
Regulated on activation, normal T cell expressed and secreted (also known as CCL5)	RANTES
Regulator T cell	T_{reg}
Renal cell carcinoma	RCC
Snail family transcriptional repressor protein 1	SNAI1
Snail family transcriptional repressor protein 2	SNAI2
Somatic copy number alterations	SCNA
Spot-forming cell	SFC
Standard operating procedure	SOP
Stromal cell-derived factor 1- α (also known as CXCL12)	SDF1- α
T cell bispecific	TCB
T cell immunoglobin mucin receptor 3	TIM3
T cell receptor	TCR
T-cell immunoglobulin and mucin-domain containing-3 (also known as HAVCR2 – hepatitis A virus cellular receptor 2)	TIM-3
Tetanus toxoid	TT
Tissue inhibitor of metalloproteinase	TIMP

Transforming growth factor	TGF
TroVax and Cyclophosphamide Treatment in Colorectal Cancer	TaCTiCC
Tumour infiltrating lymphocyte	TIL
Tumour-associated antigen	TAA
Tumour-specific antigen	TSA
Tumour, node, metastasis	TNM
Type 1 regulatory cells	Tr1
Type 3 regulatory cells	Tr3
U.S Food and Drug Administration	FDA
Vascular endothelial growth factor	VEGF
White blood cell	WBC
Zinc finger CCCH domain-containing protein 12B	ZC3H12B
Zinc finger SWIM domain-containing protein 1	ZSWIM1
zinc-finger E-box-binding protein 1	ZEB1
zinc-finger E-box-binding protein 2	ZEB2

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Chapter 1 Introduction

1.1 Colorectal Cancer

1.1.1 Incidence and Aetiology

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. There are an estimated 1.1 million new diagnoses, resulting in over 550,000 deaths per year (Jemal *et al*. 2011; Bray *et al*. 2018). The UK alone accounts for approximately 42,000 new cases. In men and women, CRC is the third most commonly diagnosed cancer, with lung cancer being the most common, and prostate/breast being the second most common in males/females respectively. It is also the second leading cause of cancer death worldwide.

Incidence of CRC is geographically varied; it is more common in economically developed countries. Moreover, incidence is higher within populations of low socioeconomic status (Doubeni *et al*. 2013). As developing countries undergo economic growth, however, incidence is becoming more prevalent. This is thought to be a reflection of evolving lifestyle and diet. There are several dietary and lifestyle risk factors associated with CRC, including intake of processed and red meat, alcohol consumption, obesity, and smoking (Liang *et al*. 2009; Chan *et al*. 2011; Fedirko *et al*. 2011; Ma *et al*. 2013; Brenner *et al*. 2014).

The risk of CRC increases with age. Additional risk factors include inflammatory bowel disease, and family history of CRC (Jess *et al*. 2012). Although most cases of CRC are sporadic, there are genetic syndromes that can increase risk, such as Lynch syndrome and familial adenomatous polyposis (FAP) syndrome (Valle *et al*. 2019). Genetic predisposition is estimated to account for 2-8% of CRC cases. Lynch syndrome occurs as a result of germline mutations in mismatch repair (MMR) genes. These genes are involved in repairing errors, such as insertions and deletions, that occur during DNA replication. Mutations in MMR genes impair their function, leading to an increase in mutated gene number and microsatellite instability (MSI). FAP occurs as a result of mutations in the adenomatous polyposis coli (APC) gene and is inherited in an autosomal dominant manner. There is also evidence that around 30% of FAP patients have no family history, thus mutations to APC can also be responsible for sporadic CRC. APC is a negative regulator of beta-catenin, which is a signal transducer in Wnt signalling; thus, APC normally functions as a tumour suppressor. FAP is characterised phenotypically by the existence of multiple adenomas.

1.1.2 Diagnosis, Staging and Prognosis

If a patient has suspected CRC, they will typically undergo a colonoscopy. During this procedure, the presence of any lesions of interest will be assessed, and biopsies taken for further investigation by histology. If CRC is confirmed, then the tumour stage will also be assessed by Tumour, Node, Metastasis (TNM) staging. This is a combination of "T" stage which relates the size of the tumour and level of penetrance through the bowel, "N" stage which assesses the involvement of regional lymph nodes, and "M" stage which discerns the presence or absence of distant metastasis. Dukes' classification is also a means of staging CRC tumours, ranging from Dukes' A – Dukes' D. This is less detailed than the TNM classification system therefore is no longer used in clinical practice in many parts of the world. TNM and Duke's staging are described and compared in detail within "Methods" (Chapter 2).

Currently, CRC can only be confirmed through colonoscopy. While this procedure is considered to be the gold standard for diagnosis, it is invasive and often poorly tolerated by patients. Moreover, the procedure requires a highly trained medical professional, and referral times can be lengthy, even when expedited. It is also important to note that the majority of patients referred for this procedure are diagnosed as cancer-free. This pathway represents a significant cost burden on the NHS. There is, therefore, an unmet need for less-invasive alternative methods for CRC diagnosis, for example a blood test. Such methods would have the potential to rapidly streamline patients for confirmatory colonoscopies.

Patient survival is significantly reduced in metastatic CRC (mCRC), as shown in Figure 1.1 (O'Connell *et al*. 2004; Arnold *et al*. 2017; Cohen and Flaherty 2018). Early identification of tumours is therefore vital to improve patient outcomes. Metastatic spread is identified by imaging methods such as Computed Tomography (CT) or Magnetic Resonance Imaging (MRI). Common sites of metastasis include liver, lungs, and peritoneum; however, the pattern of metastasis is different between rectal and colon tumours, with rectal tumours more commonly metastasizing to the lungs than colon tumours (Riihimaki *et al*. 2016). Differences are also seen in the metastatic pattern of tumours from the proximal and distal colon. This is thought to be a result of geographical lymphatic drainage.

Distant metastasis can be difficult to identify, particularly when lesions are small. Similarly, patients with earlier stage disease generally present without symptoms, making detection a significant challenge. There is, therefore, an unmet need for better

methods of disease identification. Novel blood-based approaches to identify CRC patients from healthy donors are investigated in Chapter 3 of this thesis. Although there are currently no blood-based diagnostic tests for detecting CRC used in clinical practice, carcinoembryonic antigen (CEA) in serum is frequently used to monitor treatment efficacy and relapse in known CRC patients (Wanebo *et al*. 1978; Duffy 2001; Destri *et al*. 2015).

1.1.3 Treatment

Patients without metastatic disease will typically undergo a colectomy to resect the primary tumour and affected surrounding tissue. Although this can be curative, around 50% of patients will relapse, or die from metastatic disease. After surgery, patients may receive adjuvant chemotherapy, typically a combination of several agents, for example FOLFOX (a combination of fluorouracil (5FU), leucovorin, and oxaliplatin). These drugs aim to destroy cancer cells by preventing DNA replication/synthesis. Patients are monitored over time for any indication of relapse, using methods such as colonoscopy, and imaging techniques to identify metastasis.

In patients with more advanced disease, surgery to remove the primary tumour may be performed, but this depends on the extent of disease and overall fitness of the patient. It is sometimes possible to remove metastatic lesions, particularly in the liver, although it is estimated that only 10-20% of patients with liver metastasis are eligible for this (Kim *et al*. 2010; Lintoiu-Ursut *et al*. 2015). Patients with metastatic disease will generally receive chemotherapy and/or radiotherapy in efforts to reduce symptoms and prolong life expectancy. In addition to this, there are several more specific treatment options available, for example epidermal growth factor receptor (EGFR) inhibitors such as cetuximab and panitumumab, and anti-angiogenic drugs targeting vascular endothelial growth factors (VEGF) such as bevacizumab, and aflibercept. The use of checkpoint inhibitors, such as Nivolumab and Ipilimumab, is also approved in mCRC patients with high MSI/mismatch repair deficiency and can lead to some clinical benefit (Overman *et al.* 2017; Overman *et al.* 2018).

Although there have been considerable advances in the treatment of metastatic CRC over recent years, these treatments are rarely curative. Cancer immunotherapy represents an exciting avenue for the development of novel, more personalised treatments; this will be a key focus of this thesis in Chapters 4 and 5.

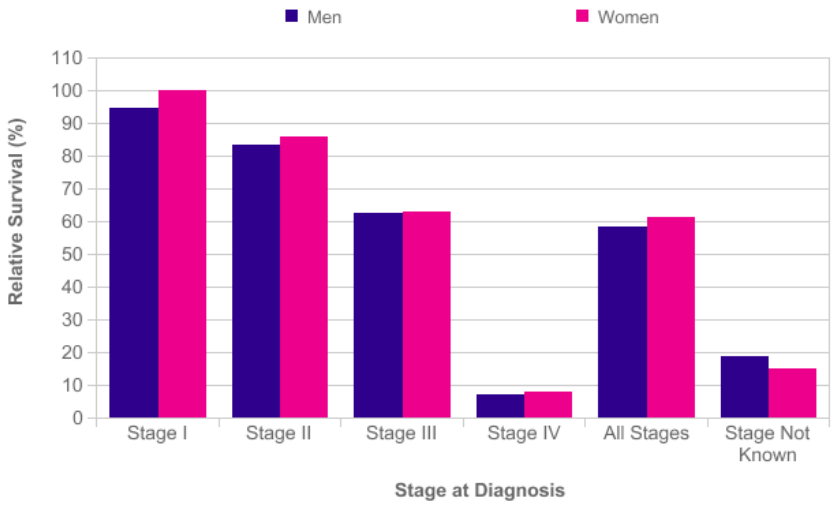


Figure 1.1 5-year survival of CRC patients by stage and gender.

This figure represents 5-year survival of patients with bowel cancer stratified by stage and gender, from 2002-2006 in the former Anglia Cancer Network. *Figure credit: Cancer Research UK.*

1.1.4 Screening

A frequent symptom of CRC is blood in stool, and this is the basis for current bowel screening methods aimed at men and women aged >55 in Wales. Small levels of blood are detected using the faecal immunohistochemical test (FIT); an abnormal result indicates potential gastrointestinal (GI) bleeding and will trigger a referral for further investigation by colonoscopy. As a result of GI bleeding, anaemia is a frequent characteristic of patients presenting with suspected CRC; this can be confirmed by full blood count (FBC) (Beale *et al*. 2005). Between 2017 and 2018, the uptake of the bowel screening programme in Wales was only 55.7%. There is, therefore, a clear need for screening methods with a higher uptake rate.

The aforementioned screening method is only routinely offered to people falling within the high-risk age group. Moreover, they are only invited to participate every two years. Individuals with family history of CRC will be offered regular colonoscopies as a screening tool. There are currently no screening options offered to individuals not within these risk groups.

1.1.5 Consensus Molecular Subtypes of Colorectal Cancer

The further stratification of cancers into molecular subtypes sharing common features has been described across cancers (Hoadley *et al*. 2014). These subtypes often relate to differences in clinical behaviour and treatment response. In CRC, the CRC Subtyping Consortium (CRCSC) was formed of a collaborative group of researchers and data scientists to understand the characterisation of the disease. This investigation culminated in a publication by (Guinney *et al*. 2015), identifying four key consensus molecular subtypes (CMS) of CRC, and a group exhibiting intermediate characteristics:

1. CMS1 (approximately 14% of cases)
   - *“Microsatellite instability (MSI) immune”*
   - CMS1 patients are defined by presence MSI, the CpG island methylator phenotype (CIMP) and hypermutation. They exhibit increased occurrence of BRAF mutations. Additionally, they have increased expression of genes associated with immune infiltration and activation and demonstrate worse survival after relapse compared to other CMS groups.

2. CMS2 (approximately 37% of cases)
   - *"Canonical"*
   - CMS2 patients have a high number of somatic copy number alterations (SCNA), in addition to increased activation of WNT and MYC signalling.

3. CMS3 (approximately 13%)
   - *"Metabolic"*
   - CMS3 patients have mixed MSI status and low SCNA and CIMP. They have increased occurrence of KRAS mutations and exhibit metabolic dysregulation.

4. CMS4 (approximately 23%)
   - *"Mesenchymal"*
   - CMS4 patients have high SCNA. Additionally, they exhibit increased stromal infiltration, TGF-β signalling, and angiogenesis. They have worse relapse-free and overall survival than other groups.

5. Other (approximately 13% of cases)
   - This represents a group of patients with mixed features and is thought to represent transitional phenotypes ("intermediate patients") or tumour heterogeneity.

Importantly, the above molecular subtyping helped to further characterise patients with "non-MSI" CRC; previously this was the key distinctive feature. The identification of these groups is useful for understanding treatment success in CRC. Patients with MSI high tumours, for example, exhibit response to immune checkpoint inhibition (Le *et al.* 2015; Overman *et al.* 2017; Overman *et al.* 2018). In addition, the usefulness of CMS classification in predicting other treatments in CRC has been investigated. Response to irinotecan (IRI)-based chemotherapy was more beneficial than oxaliplatin (OX)-based chemotherapy for CMS4 patients, and anti-EGFR receptor therapy was particularly beneficial in patients with CMS2 and led to worse survival for CMS1 patients (Okita *et al*. 2018). CMS status, therefore, may be an important consideration for precision medicine in CRC.

1.2 Tumourigenesis

1.2.1 Initiation and Establishment

The transformation of normal cells into malignant cancers is a progressive, multistep process, requiring the systematic acquisition of traits to enable tumour growth and metastatic dissemination. Six common hallmarks (1-6), and two emerging hallmarks (7-8) of this process have been described across cancers (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011), and are as follows:

1. Sustained proliferation
    - Cell growth and proliferation is largely orchestrated by growth factor signalling and is tightly regulated under normal conditions. In cancer cells, however, these processes become deregulated, leading to increased and uncontrolled cell proliferation.

2. Evasion of growth suppression
    - Under normal conditions, aberrant cellular proliferation is limited by the action of several tumour suppressor genes, for example p53. The function of such genes is impaired in cancer. Additionally, cancer cells are thought to evade anti-proliferative signals mediated by cell-to-cell contact.

3. Unlimited replicative potential
    - Healthy cells typically undergo a limited number of replications. This is in stark contrast to cancer cells, which acquire unlimited replicative potential. Telomeres progressively shorten across cellular division, ultimately triggering cell death; however, cancer cells have the ability to counter telomere erosion, thus evade destruction.

4. Resistance to cell death
    - Under conditions of stress, apoptotic/necrotic pathways are able to trigger cell death. Cancer cells become resistant to such pathways through the loss of tumour suppressor activity, for example p53, or through increased expression of anti-apoptotic proteins such as Bcl-2.

5. Induction of angiogenesis
    - Tumours, like other tissues, require influx of nutrients and oxygen and efflux of waste products to grow and survive. This is mediated by the

induction of angiogenesis via mediators such as vascular endothelial growth factors.

6. Activation of invasion and metastasis
    - Cancer cells are able to acquire invasive properties by altering the expression of cell-to-cell adhesion molecules, for example E-cadherin, and through interaction with stromal cells. Epithelial cells are also able to undergo epithelial-to-mesenchymal transition (EMT), acquiring invasive properties. Cancer cells which have disseminated from the primary tumour then undergo colonization, whereby they adapt to their new microenvironment.

7. Deregulation of cellular metabolism
    - Cancer cells alter their energy metabolism to allow for chronic proliferation. Even in the presence of oxygen, cancer cells largely favour glycolysis pathways as opposed to oxidative phosphorylation. This metabolic switch was first described by Otto Warburg, for which he was awarded the Nobel Prize in Physiology.

8. Immune evasion
    - Immune surveillance is the concept that the immune system continually monitors cells and tissues and is able to recognise and destroy cancerous/pre-cancerous cells. For cancer to progress, it must overcome these mechanisms to evade destruction, for example through creating an immunosuppressive microenvironment.

The aforementioned hallmarks of cancer are acquired as a result of two key enabling characteristics: genomic instability and mutation, and tumour-promoting inflammation. Genetic changes, such as mutational or epigenetic changes to tumour suppressors and promoters, or to key members of the DNA-maintenance machinery, underpin tumorigenesis and the acquisition of additional hallmarks. A diverse range in immune infiltration has also been described within tumours. This tumour-associated inflammatory response helps to create a pro-tumorigenic microenvironment by supplying factors required for cell growth and proliferation, angiogenesis, invasion, and metastasis. Inflammatory cells are also able to release reactive oxygen species (ROS), a known mutagen, thereby directly promoting genetic change. This thesis will primarily focus on the interaction of cancer with the immune system, and its potential for therapeutic exploitation.

1.2.2 Progression and Metastasis

Cancer metastasis remains the leading cause of death in patients. Compared to our understanding of primary tumour formation, the development of metastases remains poorly understood. The process by which a metastasis occurs is known collectively as the invasion-metastasis cascade. Briefly, this involves the primary tumour invading local tissue, followed by intravasation into blood or lymphatic vessels to facilitate transit. Cells then extravasate into distant tissues, where they first form micro-metastatic colonies, and later proliferate into metastatic lesions (Fidler 2003).

Studies have identified several mechanisms governing key events in the metastatic process. The EMT programme, for example, is able to trigger metastatic properties in epithelial cells. These include increased invasion and motility, and the ability to alter the extracellular matrix (ECM) by means of degradation (Thiery 2002). This process is triggered by surrounding tumour-associated stromal cells, including fibroblasts, endothelial cells, and immune cells, and is mediated by key transcription factors, such as snail family transcriptional repressor proteins (SNAI1 and SNAI2), and zinc-finger E-box-binding proteins (ZEB1 and ZEB2). EMT is linked to the development of cancer stem cells (CSCs), a subpopulation of undifferentiated tumour cells able to self-renew, and establish tumours (Lamouille *et al*. 2014). In CRC, EMT is mainly induced by TGF-β/SMAD and WNT/β-Catenin signalling pathways. These can increase levels of EMT transcription factors, for example SNAI1. This leads to the acquisition of EMT phenotypes such as loss of cell adhesion molecule E-cadherin (Jong *et al*. 2005; Labelle *et al*. 2011; Vu and Datta 2017). Across the board increased levels of EMT transcription factors, and loss of E-cadherin, are associated with reduced survival and increased metastasis in patients with CRC (Francí *et al*. 2009; Kahlert *et al*. 2011; Zhang *et al*. 2013; Yun *et al*. 2014). This highlights their importance in the development and metastasis of CRC.

In order for cancer cells to migrate, they must survive circulatory transport. This poses several challenges, for example, cancer cells become vulnerable to recognition by the immune system. To circumvent this, cancer cells rapidly associate with platelets, preventing recognition by natural killer (NK) cells (Kopp *et al*. 2009; Labelle *et al*. 2011). This platelet-cancer interaction has been investigated in mouse models of CRC; treatment with antiplatelet drug aspirin was able to reduce metastasis (Guillem-Llobat *et al*. 2016). In patients with previous CRC, daily aspirin led to a significant reduction in relapse (Sandler *et al*. 2003). The incidence of CRC, after 20 years of follow up, was also reduced in cohorts of patients receiving daily aspirin for the prevention of vascular

disease (Rothwell *et al*. 2010). The importance of platelets in the development of CRC is particularly highlighted by a study by (Szkandera *et al*. 2014), where it was shown that high preoperative platelet to lymphocyte ratio was predictive of reduced time to recurrence. Collectively, this evidence suggests that the platelet-cancer interaction is able to facilitate tumorigenesis and metastasis, moreover platelet count may also be a marker of systemic inflammation. Treatment with aspirin is able to reduce the pro-tumorigenic effects of platelets.

Neutrophils also promote metastasis by several mechanisms, including by directly interacting with cancer cells via secreted neutrophil extracellular traps (NETs), by secreting enzymes such as matrix metalloproteinases (MMPs) to aid intravasation and extravasation, and through direct immunosuppression of NK and T cells (Cools-Lartigue *et al*. 2013; Coffelt *et al*. 2015; Spiegel *et al*. 2016). NETs have been associated with peritoneal metastasis in CRC (Al-Haidari *et al*. 2019). Moreover, increased neutrophils levels assessed by neutrophil to lymphocyte ratio, are indicative of poor survival and increased likelihood of tumour recurrence in CRC patients (Ding *et al*. 2010; Absenger *et al*. 2013; Mallappa *et al*. 2013; Shibutani *et al*. 2013).

The ECM provides structural and biochemical support to cells and tissues and is a means of tissue organisation. It is represented by two main structures; basement membranes and interstitial matrices. The basement membrane provides a layer of separation between epithelium/endothelium and stroma, and is typically formed of type IV collagen, fibronectin, and laminins. The interstitial matrix, on the other hand, provides structural support to tissues, and is comprised of fibrillar collagens, proteoglycans, and glycoproteins such as Tenascin-C. In addition to the supportive roles of these structures, the ECM can also influence cell behaviour, both directly and indirectly, for example by binding to and regulating the distribution of growth factors (Hynes 2009). It is widely accepted that the ECM becomes deregulated in cancer, and significantly contributes to pathology, particularly aiding metastasis.

During tumorigenesis, there is increased deposition of ECM components, for example collagen, leading to tumour rigidity. This has been shown to increase integrin signalling, driving tumour progression (Levental *et al*. 2009). In CRC, the cross-linking of collagen is induced by an extracellular matrix modifying enzyme lysyl oxidase (LOX), increasing the stiffness of the matrix (Baker *et al*. 2013). Integrin signalling has been linked to the de-regulation of E-cadherin in CRC, which is associated with EMT (Avizienyte *et al*. 2002). Cancer cells can exert mechanical force and anchor cell invasion during metastasis. Additionally, in order to facilitate migration and invasion, increased

levels of ECM-degrading proteases such as MMPs are secreted, helping to disrupt basement membranes, thereby aiding metastasis. In CRC, disruption of the basement membrane is associated with increased metastasis and poor patient survival (Lazaris *et al*. 2003; Delektorskaya and Kushlinskii 2011).

Under normal conditions, such as during development and wound repair, MMPs are tightly regulated and serve to facilitate physiological matrix remodelling. During cancer development, however, MMPs and their natural inhibitors, tissue inhibitors of metalloproteinase (TIMPs), become deregulated. They contribute to the metastatic process by directly degrading the ECM, and by influencing a diverse range of cellular behaviours. MMP-7, for example, is involved in the conversion of Heparin-binding epidermal growth factor precursor (proHB-EGF) to its mature active form, thereby promoting cell proliferation. MMP-2 and MMP-9 are able to activate TGF-β, promoting angiogenesis (Yu and Stamenkovic 2000; Yu *et al*. 2002), and TIMP-1 can recruit cancer-associated fibroblasts, conferring anti-apoptotic effects (Gong *et al*. 2013; Song *et al*. 2015).

The overexpression of MMPs and TIMPs is prominent across cancer. In CRC, for example, expression levels of several MMPs and TIMPs, such as MMP-2, MMP-7, MMP-9, and TIMP-1 have been described, and associate with both metastasis and poor survival outcomes (reviewed by Said *et al*. 2014 and Crotti *et al*. 2017).

1.2.3 Cancer Immunosurveillance

As early as 1909, Paul Ehrlich proposed the idea that host natural defences are able to prevent the formation of tumours. Subsequently, several studies in mice supported this concept, including work by (Gross 1943) which demonstrated intradermal immunisation of inbred C3H mice against sarcoma, and similar work by (Foley 1953) using methylcholanthrene-induced models. This work led to the development of the "immunological surveillance" hypothesis, whereby it was proposed that immune cells are able to identify and eliminate transformed cells via the expression of "self-markers" (Burnet 1957; Burnet 1964).

This concept proved somewhat controversial at the time, with many researchers rejecting it on the basis of emerging contradictory evidence. One prominent opposing study failed to identify differences in latency or incidence of tumours between wild-type and athymic mice (Stutman 1974). Evidence subsequently emerged that the mice used

did not fully lack functional T cells, and had intact NK-mediated innate immunity, thus questioning these findings (Herberman and Holden 1978; Maleckar and Sherman 1987).

Since then, technological and scientific advances have validated the concept of cancer immunosurveillance. Evidence includes a series of studies showing that a lack of key immunological effector molecules, IFNγ and perforin, leads to increased susceptibility to spontaneous and carcinogen-induced tumours (Dighe *et al*. 1994; Kaplan *et al*. 1998; Smyth *et al*. 2000a; Smyth *et al*. 2000b). Perhaps the most striking evidence derives from a study by (Shankaran *et al.* 2001), using mice deficient in recombination-activating gene 2 (RAG-2), an enzyme required for the generation of T cells, B cells and NKT cells. Experimental exploitation of this identified that RAG-2-/- mice developed tumours quicker, and at a far greater frequency, than their wild-type counterparts. (Smyth *et al*. 2001) helped to elucidate the contributions of innate and adaptive immunity in the host defence against methylcholanthrene (MCA)-induced fibrosarcoma; both NK cells and natural killer T cells (NKT cells) were found to be important. These initial lines of evidence demonstrated a clear role for both adaptive and innate immunity in cancer immunosurveillance in mice. This concept is now integral to our understanding of human tumorigenesis.

In humans, several initial observations strongly supported the hypothesis of cancer immunosurveillance. Increased levels of virally induced cancers, such as Kaposi's sarcoma, have been widely documented across immunosuppressed patients (Gatti and Good 1971; Boshoff and Weiss 2002). Moreover, increased risk of non-viral associated cancers, for example CRC, has been described in immunosuppressed transplant patients (Birkeland *et al*. 1995), though other studies have not corroborated this. Immunosuppression may, therefore, increase tumour formation and/or or remove preventative barriers.

The ability for cancer patients to develop immune responses to tumour-specific antigens was investigated using *in vitro* autologous typing experiments whereby patient tumour cell lines were established and used to characterise antitumour immune responses (Carey *et al*. 1976). Patients were identified with antibody and T cell responses to the autologous tumour antigens (Carey *et al*. 1976; Knuth *et al*. 1984). Such responses were further characterised using gene cloning approaches, confirming both the presence of $CD8^+$ T cell responses to tumour antigens, and the ability of individuals to elicit tumour antibody responses (van der Bruggen *et al*. 1991; Sahin *et al*. 1995). It is now understood that several classes of tumour antigens are able to elicit anti-tumour

immune responses, including mutated forms of normal proteins, overexpressed normal proteins, viral proteins, and cancer-testis antigens (Vigneron 2015).

Further support for the hypothesis of cancer immunosurveillance arises from the finding that the presence of tumour-infiltrating lymphocytes (TILs) correlates with patient survival across several cancers (Clemente *et al*. 1996; Naito *et al*. 1998; Zhang *et al*. 2003; Gooden *et al*. 2011). Patients with higher numbers of $CD3^+$ and $CD4^+$ TILs had improved overall and progression-free survival. In CRC, this concept was been explored in detail by (Galon *et al.* 2006). In this study, immunostaining was performed on the primary tumours of 415 CRC patients to identify the number and type of tumour infiltrating immune cells. Stage I-III patients with low numbers of infiltrating CD3 cells, or with low levels of CD3 and CD45RO cells, had significantly reduced disease-free survival compared to those with high numbers. Importantly, this had superior prognostic value that traditional TNM staging for these patients, implying a vital role for adaptive immune responses in CRC.

1.2.4 Cancer Immunoediting

As described, there is a wealth of evidence to support that the immune system is able to confer a degree of protection against cancer. Conversely, there is also evidence that by exerting this function, the immune system is able to select for tumours of low immunogenicity, thereby also promoting tumorigenesis. This led to the cancer immunosurveillance hypothesis evolving into the theory of "cancer immunoediting" whereby the interaction of the immune system with cancer is defined by three key stages; elimination, equilibrium, and escape (Dunn *et al*. 2004; Schreiber *et al*. 2011). Cancer immunosurveillance therefore represents the first stage in a much more complex process.

During the equilibrium phase, tumour cells that have evaded immune destruction are controlled by the competent immune system, through the continuous pressure of IFNγ. Convincing anecdotal evidence for cancer cell dormancy exerted by the immune system comes from a report by (MacKie *et al.* 2003) describing the occurrence of metastatic melanoma in two renal transplant patients. These patients both received a kidney from the same donor; an individual who had previously been diagnosed with melanoma and was, at her time of death, deemed cancer-free for over 15 years. It is likely that the donor's competent immune system was able to maintain tumour cells in a state of dormancy, and this was lost during immunosuppression in organ transplant recipients. This hypothesis has been tested in mouse models, generating convincing

supportive evidence. One prominent study by (Koebel *et al.* 2007) investigated the outcome of MCA-induced tumours in immunocompetent mice, with or without monoclonal antibodies to deplete $CD4^+/CD8^+$ cells, and to neutralise IFNγ/IL-12. Depletion of $CD4^+/CD8^+$ cells, or neutralisation of IFNγ/IL-12 led to tumour outgrowth in these mice, compared to those treated with control immunoglobin. There was found to be no difference in tumour outgrowth when NK cell function was blocked, highlighting the importance for adaptive immunity in the equilibrium phase.

The final phase, escape, encompasses aspects of the equilibrium phase whereby selective pressure applied to tumour cells to maintain them in a state of dormancy also promotes the outgrowth of less immunogenic clones. Effective tumour immunosurveillance requires the combined efforts of the innate and adaptive immune system, therefore in order to escape detection, tumour cells must bypass both systems. This can occur in several ways, including; loss of antigen processing function, reduced/loss of expression of antigens, and loss of major histocompatibility complex (MHC) class 1, which is required for presentation of antigens to T cells, and IFNγ insensitivity. Additionally, the creation of an immunosuppressive microenvironment conducive to tumour growth can promote escape. This is mediated by the production of immunosuppressive cytokines, for example transforming growth factor-β (TGF-β) and indoleamine 2,3-dioxygenase (IDO), and/or through the recruitment of suppressive immune cells, such as regulatory T cells (T_{reg}) or myeloid-derived suppressor cells (MDSCs) (Vesely *et al*. 2011). T_{reg} cells and their implications for cancer immunotherapy is a key focus of this thesis, therefore is discussed later in more detail.

1.3 T Cells

1.3.1 Overview of Innate and Adaptive Immunity

Protection against invading pathogens is mediated by the innate and adaptive arms of the immune system. The former represents the first line of defence and consists of a wide range of cell types; macrophages, neutrophils, basophils, eosinophils, dendritic cells (DCs), and NK cells. Many of these cells possess pathogen recognition receptors (PRRs) which are able to recognise pathogen-associated molecular patterns (PAMPs); PAMPs are commonly conserved patterns which are not present on host cells, thereby allowing innate immune cells to distinguish between "self" and "non-self". Some well-known examples of PAMPs include bacterial cell surface carbohydrates such as peptidoglycans and lipopolysaccharides, bacterial proteins such as flagellin, and bacterial and viral nucleic acids.

The stimulation of the innate immune system initiates adaptive immunity. This is more specific and is mediated by the actions of B and T cells. B cells are primarily responsible for the production of antigen-specific antibodies in response to antigen recognition through the B cell receptor (BCR), however also function as professional antigen presenting cells (APCs). T cells are able to recognise antigens bound to MHC molecules via the T cell receptor (TCR). Several functionally distinct groups of T cells exist; therefore, antigen recognition leads to myriad of downstream pro- and anti-inflammatory effects.

1.3.2 T Cell Subsets

As described, there are a range of T cell subsets as summarised in Figure 1.2. This allows the generation of distinct and diverse immune responses. To simplify this, T cells are grouped by function into:

1. $CD4^+$ T cells ("helper T cells").
2. $CD8^+$ T cells ("cytotoxic T cells").
3. Memory T cells.
4. Regulatory T cells (T_{reg} cells).
5. T cells with innate-like functions e.g. NKT cells, gamma delta (γδ)T cells, and mucosal associated invariant T cells (MAIT cells).

CD4 and CD8 T cells are the main effector populations, defined by the presence of glycoproteins cluster of differentiation 4 and 8 (CD4 and CD8) on their cell surfaces. These proteins function as TCR co-receptors, allowing the interaction with peptides presented by MHC class II and class I respectively.

$CD8^+$ cytotoxic T cells, upon recognition of antigen, function to kill infected or transformed cells. This occurs through the release of cytotoxic granules containing destruction mediators such as granzyme B and perforin, through the release of cytokines such IFNγ and TNFα, and through Fas/FasL interactions.

$CD4^+$ helper T cells are required for both host defence and for immune homeostasis. They differentiate into two main subtypes; Th1 and Th2 cells, however other subtypes have also been identified, such as Th17 cells. Each subtype has unique functional properties and exerts these through secreted pro- and anti-inflammatory cytokines. Th1 cells are involved in the defence against intracellular pathogens and are defined by their secretion of IFNγ and IL-2, however also secrete other cytokines such as TNF-α, and lymphotoxin. These cytokines have a range of downstream effects, for example, IFNγ leads to the activation of macrophages, and IL-2 promotes T cell differentiation and proliferation. The differentiation of naïve T cells into Th1 cells is largely orchestrated by IL-12, IFNγ and IL-2. Th2 cells are important for the defence against extracellular parasites, for example helminths, and are defined by their secretion of IL-4, IL-5 and IL-13, in addition to IL-9 and IL-10. The differentiation of naïve T cells into Th2 cells mediated by IL-4 and IL-2.

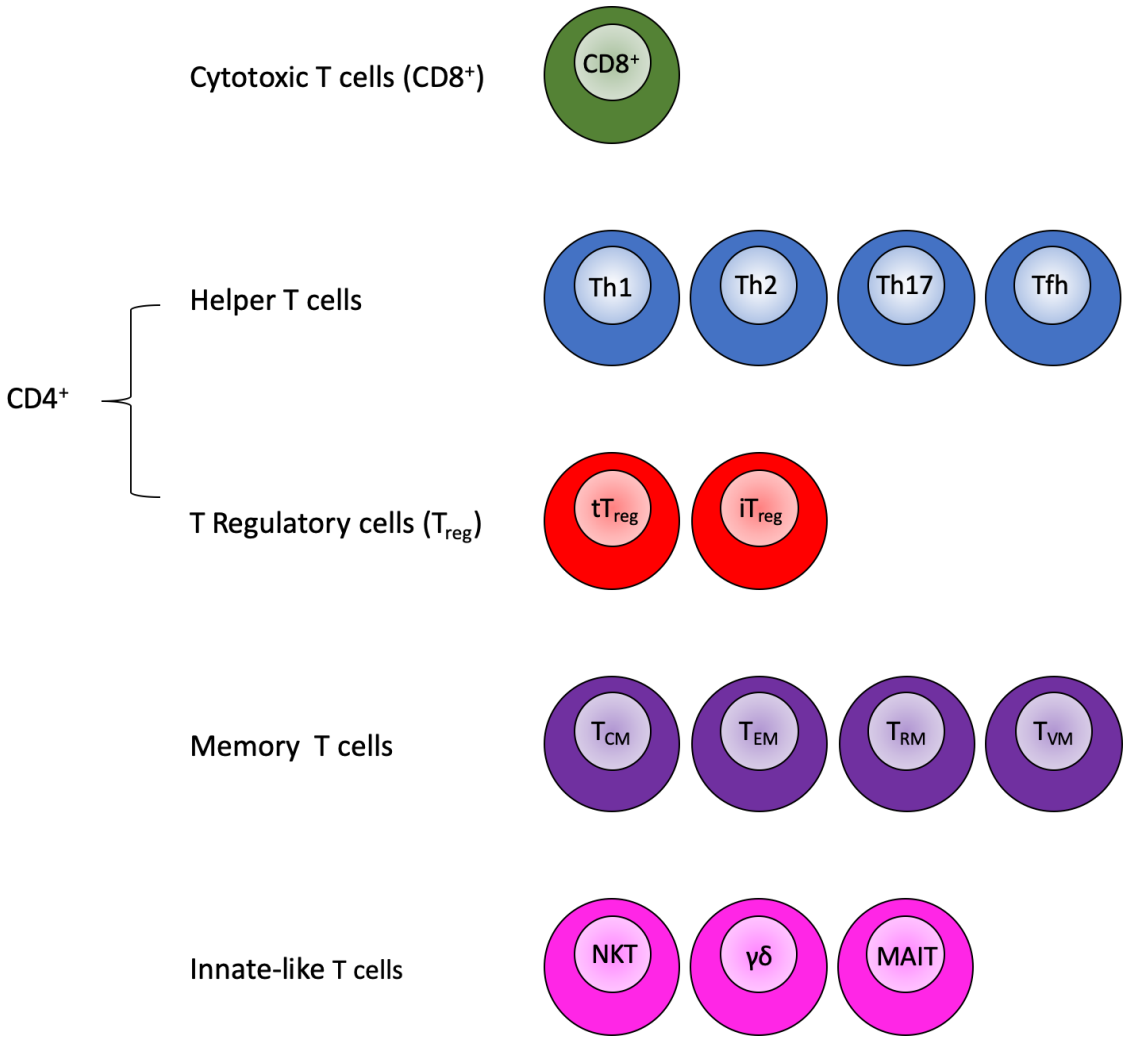


Figure 1.2 Overview of Key T Cell Subsets.

This figure shows a diagrammatic overview of the key T cell subsets; cytotoxic, helper, regulatory, memory, and innate-like. Within these subsets there are often several functionally and phenotypically distinct subclasses. Helper T cells typically differentiate into Th1 or Th2 cells, however there are also other subclasses such as Th17 and T follicular helper (Tfh) cells. Regulatory T cells are typically classed as either thymically-derived (tTreg) or induced (iTreg). Classically memory T cells differentiate into two main populations; central memory (TCM), effector memory (TEM), however, other subtypes have also been described, for example resident memory (TRM), and virtual memory (TVM). Innate-like T cells encompasses a diverse range of T cells with innate-like functions, such as natural killer T (NKT) cells, γδ T cells, and mucosal associated invariant T (MAIT) cells. This is a broad overview of T cells subsets; therefore, it should be noted that several other subclasses have also been described.

1.3.3 Regulatory T Cells

Regulatory T cells (T_{reg} cells) are a subset of T cells able to control and limit immune responses to both foreign and self-antigen. The existence of a subset of cells able to limit immune activity, and therefore protect against autoimmunity, was first suggested by (Gershon *et al.* 1972), then later by (Powrie and Mason 1990). This regulatory subset was convincingly identified as being $CD4^+CD25^+$ T cells by (Sakaguchi *et al.* 1995); mice depleted of these cells developed autoimmune disease which could be prevented by reconstitution. Studies in mice and humans have confirmed that these $CD4^+CD25^+$ cells are able to suppress T cell activation through inhibiting IL-2 production (Thornton and Shevach 1998; Ng *et al*. 2001; Stephens *et al*. 2001).

Through subsequent investigation, it has been shown that T_{regs} mediate immunosuppression in a variety of manners. They are able to produce a range of inhibitory cytokines, for example TGF-β and IL-10, in addition to cytolysis-mediating granzyme B. Moreover, they are able to interact with DCs, reduce antigen presentation and cytokine production, and can cause metabolic disruption.

In 2003, two key studies identified forkhead transcription factor 3 (Foxp3) as a critical mediator of T_{reg} development and function (Fontenot *et al*. 2003; Hori *et al*. 2003). In the study by Fontenot *et al*, Foxp3 deletion or mutation prevented the generation of T_{reg} cells, resulting in lethal autoimmunity in mice. Foxp3 mutation results in the "scurfy" phenotype in mice characterised by extensive T cell proliferation and multiorgan infiltration leading to extensive associated pathology such as enteropathy, diabetes, and dermatitis (Brunkow *et al*. 2001). This phenotype is also demonstrated in Foxp3 null mice and could be rescued by the addition of functional $CD4^+CD25^+$ cells (Fontenot *et al*. 2003). Hori *et al* clearly demonstrated the role of Foxp3 in the development of T_{reg} cells through ectopic retroviral transduction of Foxp3 in $CD25^-CD4^+$ cells; these cells developed into T_{reg} cells, were able to suppress T cell proliferation *in vitro*, and inhibited the development of inflammatory bowel disease (IBD) in mouse models. In humans, a syndrome causing similar pathology to "scurfy" in mice was identified; IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome (Kim *et al*. 2001). Sequencing of the Foxp3 gene in these patients identified several mutations leading to Foxp3 inactivation and therefore an impairment in the generation of T_{regs} (Bennett *et al*. 2001; Kim *et al*. 2001). These studies suggested that Foxp3 plays an important role in the generation of function T_{reg} cells, which are in turn able to maintain immune homeostasis.

Prior to the discovery of Foxp3 as a key mediator of T_{reg} function, there was a lack of markers for identifying T_{regs}. The majority of T_{reg} cells are naturally occurring, thymically derived, and are defined as $CD4^{+}CD25^{+}Foxp3^{+}$ cells. It is also possible, however, to generate "peripherally induced" T_{regs} which generally do not express Foxp3. These populations include type 1 and 3 regulatory cells (Tr1 and Th3 cells). Tr1 cells typically produce IL-10 in order to confer their suppressive function (Vieira *et al*. 2004; Roncarolo *et al*. 2006), whereas Th3 cells produce TGF-β (Weiner 2001). Since then, the classification and nomenclature of these cells has evolved, with an agreement on thymically derived T_{regs} and peripherally derived/induced T_{regs} (Abbas *et al*. 2013). Foxp3 is therefore not a definitive marker of T_{regs}, however is still used for identification in combination with CD4 and CD25, due to its known role in T_{reg} development and function. This poses several difficulties; there are subsets of T_{regs} which do not express Foxp3, Foxp3 is intracellular, therefore cannot be used for the isolation of live T_{reg} cells, and both Foxp3 and CD25 are also known to be increased on activated non-regulatory T cells (Kmieciak *et al*. 2009). This led to the identification of several other markers of T_{reg} function, including; reduced expression of CD127, and increased expression of CD39/CD73, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), inducible costimulator (ICOS), and Helios (Wing *et al*. 2008; Camisaschi *et al*. 2010; Thornton *et al*. 2010; Vocanson *et al*. 2010; Yu *et al*. 2012).

There has been much interest in understanding T_{reg} function, and how this is implicated in health and disease. The current understanding of T_{reg} function is extensively reviewed by (Zhao *et al.* 2017). Research has defined a clear role for these cells in the control of immune homeostasis; they can prevent autoimmune disease. In addition, T_{reg} cells have been investigated in the context of cancer. The role of T cells, both effector and regulatory, in the development and treatment of CRC is a key focus of this thesis and will be explored below.

1.4 T Cells and Colorectal Cancer

1.4.1 Tumour Infiltrating Lymphocytes in Colorectal Cancer

The association with increased immune cell infiltration in solid tumours, and its positive association with survival was first described by (MacCarty 1931). Since then, this has been observed and confirmed across several cancers, including in melanoma, ovarian, and colorectal cancers (Clark *et al*. 1989; Ropponen *et al*. 1997; Zhang *et al*. 2003).

In CRC, TILs were identified as independent predictors of overall and recurrence-free survival (Ropponen *et al*. 1997). As previously described, the effect of immune cell infiltration was further characterised by Galon *et al* in 2006. This study identified that CRC patients with high numbers of infiltrating $CD3^+$/$CD3^+CD45RO^+$ T cells had improved survival. Strikingly, immune infiltration was shown to be a better predictor of patient outcome than traditional TNM measurements; Stage I-III patients with high infiltration had significantly improved survival than those with low immune infiltration. This study provided strong evidence that adaptive immunity can exert tumour control. Moreover, it clearly demonstrated that survival outcomes in patients with the same TNM stage (Stage I-III) are varied; this may be important for understanding treatment outcome. In light of these landmark findings, an international consortium was established to promote and harmonize the use of immune $CD3^+$ and $CD8^+$ T cell infiltration ("Immunoscore") in the clinical setting (Galon *et al*. 2012). The usefulness of the consensus Immunoscore on predicting tumour recurrence has subsequently been validated across 14 centres in 2681 CRC patients (Pagès *et al*. 2018).

1.4.2 Regulatory T Cells and Colorectal Cancer

The number of regulatory T cells within the tumour has been proposed as a mechanism for tumour immune suppression. In CRC, the number of peripheral and intratumoral T_{regs} is significantly increased compared to healthy donors (Clarke *et al*. 2006; Ling *et al*. 2007), a finding that is mirrored across several other cancers (Wolf *et al*. 2003; Ormandy *et al*. 2005).

The proportion of peripherally circulating T_{regs} has been shown to increase in CRC patients with later stage disease, defined by TNM staging (Scurr *et al*. 2013). Moreover, increased peripheral T_{reg} numbers are able to suppress anti-tumour immune responses, leading to CRC progression (Betts *et al*. 2012; Scurr *et al*. 2013). These results support

that T_{regs} are able to impinge upon anti-tumour immunity, potentially by induction of an immunosuppressive microenvironment, and that they may represent an important target for immunotherapy.

There is, however, some controversy with regards to tumour infiltrating T_{reg} number and CRC prognosis, with some studies suggesting that increased numbers are in fact protective. (Salama *et al.* 2009), for example, demonstrated improved survival associated with increased $Foxp3^+$ T_{regs}. A potential explanation for this could be that T_{regs} are also important to resolve cancer-promoting inflammation, and hence may play an important role in the early stages of cancer.

1.4.3 Tumour-Associated Antigens

The adaptive immune system may exert control over tumorigenesis through the recognition of tumour antigens. Tumour antigens are broadly classified into those expressed exclusively by tumour cells and those which are present on tumour cells and some normal cells; tumour-specific antigens (TSAs) and tumour-associated antigens (TAAs). Within these groups, several more defined classes of antigen exist, each with varying degrees of tumour specificity (Coulie et al. 2014; Vigneron 2015; Finn 2017). Antigens with high specificity exhibit a tumour-specific expression pattern. These include:

1. Viral antigens, for example those derived from human papillomavirus (HPV).
2. Antigens encoded by mutated genes, for example cyclin-dependant kinase 4 (CDK4).
3. Cancer-testis/oncofoetal antigens. These are expressed in tumours, with normal expression restricted to male germ cells, or to foetal development. Examples include melanoma-associated antigen 1 (MAGEA1), New York oesophageal squamous cell carcinoma 1 (NY-ESO-1), and 5T4.

Tumour-associated antigens have lower tumour specificity, and often show levels of background expression. These include:

1. Differentiation antigens. These are expressed both in the tumour and the corresponding heathy tissue. Examples include melanoma antigen recognised by T cells 1 (MART-1), carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), and tyrosine.

2. Overexpressed antigens. These antigens show increased expression in tumours compared to healthy tissue. Examples include mucin 1 (MUC1), cyclin B1, and human epidermal growth factor receptor 2 (HER2/neu).

In CRC, two key antigens of interest have been largely explored within the Godkin/Gallimore research group; 5T4 and CEA. 5T4 is an oncofoetal antigen and CEA is an overexpressed antigen, both of which are upregulated on a high proportion of colorectal tumours, with limited expression on healthy tissue (Davidson *et al*. 1989; Starzynska *et al*. 1992). This observation led to a detailed investigation into the immunogenicity of these antigens, and their potential as immunotherapeutic targets; this is further explored in section 1.4.4.

1.4.4 Anti-Tumour Immune Responses in Colorectal Cancer

The Godkin/Gallimore group is interested in understanding the role of anti-tumour immune responses in the context of CRC. This has been extensively investigated in relation to oncofoetal antigens 5T4 and CEA, based on the observation that they are overexpressed in a high proportion of CRC tumours.

Ex vivo 5T4-specific $CD4^+$ T cells responses have been described in the PBMC of CRC patients and healthy donors, and have been shown to increase in CRC patients after surgery (Clarke *et al*. 2006; Betts *et al*. 2012). Suppressed responses associated with progression and tumour recurrence. These responses could be increased upon T_{reg} depletion, suggesting that T_{regs} may be reducing anti-tumour immune responses (Betts *et al*. 2012). This was further assessed by Scurr *et al* 2013 by enumerating cultured T cell responses to 5T4 in pre-operative CRC patients and healthy donors. Robust cultured 5T4 responses were found in all healthy donors, whereas response to 5T4 was significantly reduced in CRC patients. Responses in CRC patients were generally increased after tumour resection. Moreover, there was a significant reduction in 5T4 responses based on tumour staging and this correlated with T_{reg} number, providing clear rationale for the depletion of T_{regs} in CRC immunotherapy. This was tested through treatment of Stage IV CRC patients with low-dose cyclophosphamide (CPM), a chemotherapeutic able to selectively deplete T_{reg} cells at low doses; after treatment 5T4 T cell responses were significantly increased (Scurr *et al*. 2013). This led to a phase II clinical trial to investigate the effect of CPM treatment and/or 5T4 cancer vaccination on anti-tumour immune responses in stage IV CRC patients; this will be discussed in detail in the subsequent section entitled “TroVax and Cyclophosphamide Treatment in Colorectal Cancer” (TaCTiCC). Interestingly, the presence of CEA-specific T cell

responses has also been observed in CRC patients. This was, however, significantly related to increased tumour recurrence, suggesting that not all immune responses are beneficial (Scurr *et al*. 2015).

1.5 Cancer Immunotherapy

1.5.1 Overview of Cancer Immunotherapy

As previously described, the immune system represents a fundamental barrier in tumorigenesis and metastasis and is frequently suppressed by the tumour. In light of this, cancer immunotherapy aims to target the immune system, through promoting effective anti-tumour immunity and reducing tumour-mediated immunosuppression, in order to treat cancer. This area of research has grown exponentially in recent years and has demonstrated striking therapeutic benefit and increased survival in some patients. This led to cancer immunotherapy being named “Breakthrough of the Year” by Science magazine in 2013.

The successful use of immunotherapy for the treatment of cancer was documented as early as 1893, with William Coley describing tumour regression in patients with inoperable tumours in response to inoculation with heat-inactivated bacteria (Coley 1893; Coley 1908). This treatment coined the name “Coley’s toxins”. Further support for this concept was provided by a study showing the positive effect of Bacillus Calmette-Guérin (BCG) on the treatment of bladder cancer (Morales *et al*. 1976).

Following on from the discoveries by Coley and Morales, and in light of emerging evidence for tumour immunosurveillance, new methods of treating cancer using immunotherapy were investigated. Early methods included the systemic treatment of patients with immunostimulatory cytokines, such as IL-2. The administration of IL-2 was shown to induce tumour regression in patients with metastatic renal cell carcinoma (RCC) and metastatic melanoma (Rosenberg *et al*. 1985), leading to its approval for treatment by the U.S Food and Drug Administration (FDA) in 1992 and 1998 respectively. Unfortunately, response rates to IL-2 were low and its benefit was confined to a subset of cancers. Moreover, patients frequently experienced adverse side effects. This led to the development of more targeted and effective approaches for cancer immunotherapy such as cancer vaccination, adoptive cellular transfer, and immune checkpoint inhibition.

Adoptive cell therapy involves the isolation of patient tumour-specific T cells, *ex vivo* expansion, followed by infusion back into the patients. This approach is thought to

bypass the requirement of breaking immune tolerance by directly providing anti-tumour T cells. In patients with metastatic melanoma, this approach demonstrated durable responses and complete tumour regression in 20/93 patients (Rosenberg *et al*. 2011). Although this may at first appear modest, complete responders had a 5-year survival of 93% representing a significant improvement on previous 5-year survival rates in this patient cohort of only 5%. More recently, adoptive T cell transfer has been enhanced by the generation of chimeric antigen receptor (CAR) T cells. Using this methodology, patient T cells are genetically modified to express a combination of antigen-binding domains derived from variable domains of antibodies, TCR signalling domains, and costimulatory domains. This allows CAR T cells to recognise antigen independently of MHC. Thus far CAR T cell therapy has proven effective in the treatment of B cell malignancies through targeting frequently overexpressed CD19 on the cell surface (Porter *et al*. 2011). Two therapies have currently been approved by the FDA for this purpose; Tisagenlecleucel and Axicabtagene ciloleucel. These treatments are, however, prohibitively expensive and often lead to significant side effects such as cytokine release syndrome. Moreover, tumour-escape mechanisms can lead to loss of CD19 expression and subsequent relapse. So far, these therapies have only demonstrated clear success in the treatment of haematological cancers.

The development of immune checkpoint inhibitors arguably represents the largest advancement in cancer research in recent years. For their contribution to this, James Allison and Tasuku Honjo were awarded with the Nobel Prize in Physiology and Medicine in 2018. Checkpoint inhibitors work to block immunosuppressive mechanisms which are activated by the cancer cell, in order to remove the barriers controlling anti-tumour immune responses. One such immunosuppressive mechanism is orchestrated by CTLA-4; CTLA-4 is constitutively expressed on T_{reg} cells and becomes upregulated on conventional T cells upon TCR engagement. It acts to dampen immune responses by competing with CD28 for binding of CD80 and CD86 on the surface of APCs. CTLA-4 has greater affinity and avidity for CD80 and CD86 than CD28. It is also able to directly deplete the levels of its ligands through trans-endocytosis, thereby inhibiting CD28-mediated T cell costimulation, and further contributing to its immunosuppressive function (Qureshi et al. 2011). Ipilimumab, an anti-CTLA-4 monoclonal antibody, was first approved by the FDA in 2011 for the first-line treatment of metastatic melanoma after demonstrating improved survival during clinical studies (Hodi *et al*. 2010). Since then, additional checkpoint inhibitors have been developed to disrupt the programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) interaction, including nivolumab and pembrolizumab which are anti-PD-1 monoclonal antibodies, and atezolizumab which is an anti-PDL-1 monoclonal antibody. PD-1 becomes expressed on

activated T cells and interacts with its ligand PD-L1, which is widely expressed across non-lymphoid tissues, to attenuate local T cell responses. PD-L1 is frequently overexpressed on cancer cells, an represents a clear mechanism by which anti-tumour immunity is impeded. Combination therapy with Nivolumab plus Ipilimumab has been shown to induce higher responses in metastatic melanoma patients, with 36% demonstrating complete responses, and over 50% of patients at least partially responding (Larkin *et al*. 2015).

Since the development of checkpoint inhibitors, their efficacy had been tested across several cancers, leading to frequent FDA approval for treatment. Despite this, responses are only demonstrated in a minority of patients, and treatment often leads to serious adverse immune-related side effects. Moreover, response to immunotherapies has thus far been far more efficacious in a subset of cancers, such as melanoma. New treatment methods are required, and this is an area of intense research. Immunotherapy advances in the context of CRC will be discussed below.

1.5.2 Immunotherapy in Colorectal Cancer

Immune checkpoint inhibition with PD-1 blocking antibodies nivolumab and pembrolizumab, and combination therapy with nivolumab and CTLA-4 blocking antibody ipilimumab, gained accelerated FDA approval in 2017 for the treatment of patients with high microsatellite instability (MSI-H) or mismatch-repair-deficient (dMMR) metastatic CRC. Treatment of patients with microsatellite-stable (MSS) or mismatch-repair-proficient (pMMR) metastatic CRC, however, has not proven efficacious.

In a phase 2 study by Le at al. in 2015, pembrolizumab was administered to dMMR and pMMR mCRC patients. Immune-related responses and subsequent progression-free survival were demonstrated in a proportion of dMMR patients, but not in pMMR patients (Le *et al*. 2015). These findings prompted the phase 2 clinical trial Checkmate 142, to investigate the efficacy of nivolumab monotherapy and combinational therapy in metastatic dMMR/MSI-H CRC patients. Although still ongoing, results thus far have been encouraging. Nivolumab monotherapy induced responses in 23/74 patients, with 68.9% controlling disease for 12 weeks. 12-month overall survival was 73% (Overman *et al.* 2017). In combinational Nivolumab plus Ipilimumab, these results were even better, with an objective response rate of 55%, and 12-month progression-free and overall survival in 71% and 85% of patients respectively (Overman *et al.* 2018).

The disparity in response between MSI-H/dMMR and MSS/pMMR mCRC patients is thought to be a result of mutational load and immune cell infiltration; MSI-H/dMMR tumours are highly mutated and have high numbers of infiltrating immune cells, whereas MSS/pMMR tumours have a low number of mutations and exhibit low levels of immune infiltration. This hypothesis is supported by a study comparing the mutational landscape across cancers; those with the highest mutational load were melanoma and non-small-cell lung cancer (NSCLC), the cancers which show the strongest benefit from immune checkpoint inhibition (Alexandrov *et al*. 2013). Moreover, dMMR status was also shown to be predictive of response to PD-1 blockade across 12 other tumour types (Le *et al*. 2017) It is believed that tumours with high mutational burdens present increased numbers of neoantigens, therefore are more likely to be recognised by the host immune system, particularly when immunosuppression is alleviated. Checkpoint inhibition is a fantastic example of the usefulness of CMS classification for stratifying patients; CMS1 tumours are defined as MSI high with high infiltrating immune cells, therefore are prime candidates for treatment.

Considering that the vast majority of mCRC patients are MSS/pMMR, the lack of efficacy of current immunotherapies in this cohort represents a significant challenge. The fundamental reason is related to lack of immune infiltration into the tumour. In efforts to achieve clinical responses, investigations into combinational therapies to simultaneously inhibit immune checkpoints and increase anti-tumour immune infiltration are ongoing. One such approach is the use of T cell bispecific antibody CEA-TCB in combination with PD-L1 inhibition. This antibody binds to both upregulated CEA on CRC tumour cells and to CD3, leading to T cell engagement and activation. Although still ongoing, this study has yielded somewhat promising results, with a response rate of 18%, and disease control of 82% (Segal *et al.* 2017a). This is the first bi-specific antibody to show efficacy in solid tumours. A multitude of investigations are currently underway into combinational approaches to treat MSS/pMMR mCRC patients. These include combinations of chemotherapy/radiotherapy, and checkpoint inhibition, frequently in addition to targeted treatments such as MEK and VEGF inhibition. Moreover, alternative immunotherapeutic approaches are being investigated, such as inhibition of other T cell checkpoints like T cell immunoglobin mucin receptor 3 (TIM3) and LAG-3, adoptive cellular transfer, and vaccination. This is comprehensively reviewed by (Ganesh *et al*. 2019). Furthermore, there are ongoing efforts to identify biomarkers predicting patients who are likely to respond to therapy, such as mutational burden, in addition to biomarkers of objective response within these patients. The Godkin/Gallimore group has recently investigated the use of 5T4 cancer vaccination TroVax and/or T_{reg} depletion by CPM in mCRC

patients and demonstrated promising immunological responses and survival outcomes. This will be discussed in detail in section 1.5.4.

1.5.3 Cancer Vaccines

The discovery and characterisation of tumour antigens has led to widespread interest in the development of therapeutic cancer vaccines. Initially research focussed on peptide-based vaccines, however, as the field has grown, alternative strategies have also been investigated, for example using dendritic cell-based vaccines, using viral vectors, targeting multiple antigens, or using vaccines in combination with other therapies. Although there has been significant research into developing these vaccines, the resultant products have thus far proven ineffective at prolonging overall and progression-free survival, and preventing disease recurrence (Finn 2018).

To date, only one therapeutic vaccine has been approved by the FDA; Sipuleucel-T for the treatment of castration-resistant prostate cancer. This approval was based on a 4.1 month increase in median survival, further highlighting the limited success of this therapeutic strategy (Kantoff et al. 2010). Treatment with Sipuleucel-T costs approximately £50,000 per patient, therefore in the UK the National Institute for Health and Care Excellence (NICE) do not support its use.

Although treatment with cancer vaccines has thus far shown limited success, there has been considerable progress in utilising such vaccines for cancer prevention. In particular, preventative vaccination campaigns against cancer-associated viruses human papillomavirus (HPV) and hepatitis B virus (HBV) have shown promise. Such vaccines act to prevent infection meaning that the risk of developing associated cancers, such as cervical cancer, HPV-positive oral cancer, and hepatocellular carcinoma (HCC), is greatly reduced. In Taiwan, a HBV immunisation programme has led to a significant reduction in HCC incidence in immunised individuals (Chang et al. 2016). Similarly, since its introduction in the mid-2000s, HPV vaccination has led to a significant reduction in infection (Markowitz et al. 2016); it is anticipated this reduction in HPV prevalence will longitudinally translate into a significant reduction in cases of HPV-associated cancer. Remarkably, clinical responses have been demonstrated in patients with pre-malignant vulvar and cervical lesions upon vaccination with HPV-derived peptides (Finn and Edwards 2009; Kenter et al. 2009). This suggests that effective cancer treatment may be possible using a cancer vaccine, however, it may currently be a more appropriate in the context of cancer prevention, or early-disease treatment.

Currently, trials are also ongoing to investigate the efficacy of preventative vaccines for non-viral associated cancers. One such vaccine is against overexpressed tumour antigen MUC1; on the basis of a promising feasibility study, this is currently being assessed as a preventative CRC cancer vaccine in a cohort of 110 patients with a history of advanced villous adenomas (Kimura et al. 2013). It is hoped that this vaccine will prevent the development of pre-malignant lesions into cancer. Moreover, such vaccines may be investigated for their potential prophylactic use in high risk groups.

It could be hypothesised that the limited efficacy demonstrated by therapeutic vaccines in patients with cancer may be a result of disease stage. Early-stage clinical trials are undertaken in patients with advanced disease who have typically exhausted other treatment options; this may simply be too late to observe efficacy and/or survival benefits. This hypothesis also supports the comparative success seen in the preventative setting, and in those patients with pre-malignant lesions. The Godkin/Gallimore group has previously shown that in the number of T_{regs} is significantly increased in patients with advanced CRC compared to both earlier-stage patients, and to healthy donors (Betts et al. 2012; Scurr et al. 2013). These T_{regs} impinge upon effective antitumour immunity and contribute to the immunosuppressive tumour microenvironment. It is possible that in patients with advanced cancers, that this elevated immunosuppression is a significant barrier in achieving clinical responses to cancer vaccines. It could therefore be argued that cancer vaccine efficacy and response could be improved by simultaneously alleviating tumour-associated immunosuppression. This concept was tested in CRC patients by the Godkin/Gallimore group in a recent clinical trial and is described in more detail in section 1.5.4.

1.5.4 TroVax and Cyclophosphamide Treatment in Colorectal Cancer (TaCTiCC)

Recently the Godkin/Gallimore group published the findings from the clinical trial "TroVax and Cyclophosphamide Treatment in Colorectal Cancer" (TaCTiCC). TaCTiCC (Scurr *et al*. 2017a; Scurr *et al.* 2017b). This trial investigated the effect of low-dose CPM and/or 5T4 cancer vaccine TroVax, on the induction of anti-tumour immune responses in patients with advanced mCRC. The rationale behind this was to alleviate T_{reg}-mediated immunosuppression using low-dose metronomic CPM, and to promote anti-tumour immune responses to 5T4, an antigen commonly expressed on CRC tumours, using TroVax. 52 patients were split into the following arms:

1. Watch and Wait (n=8)
2. CPM treatment (n=9)
3. TroVax treatment (n=17)
4. Combined CPM and TroVax treatment (n=18)

CPM treatment was able to deplete T_{reg} numbers and increase IFNγ T cell responses to 5T4. Additionally, 5T4 vaccination significantly increased 5T4 antibody levels, and was able to induce 5T4-specific IFNγ T cell responses.

Although the primary endpoint of the trial was enhanced immunological responses, survival benefits were also demonstrated. Significantly increased progression-free survival was seen in all treated groups combined compared to controls. Additionally, when magnitude of T_{reg} depletion was used to assess response to CPM; patients who responded to therapy had improved progression-free survival compared to those who did not (5 months versus 2.5 months). Similarly, patients who responded to TroVax treatment, as determined by increased magnitude of 5T4-specific IFNγ T cell and antibody response, had significantly improved progression-free and overall survival (5.6 versus 2.4 months, and 20 versus 10.3 months respectively). The survival curves are shown in Supplementary Figures 1-5 of this thesis. Importantly, there was no additional increase in survival demonstrated in the group receiving dual therapy; both CPM and TroVax resulted in similar survival benefits, and this is not further increased by combination.

The observed survival benefits were somewhat unexpected considering the advanced nature of disease in the cohort. In light of the results, however, both CPM and TroVax represent potentially useful immunotherapies for advanced mCRC. CPM and TroVax were safe and well tolerated in patients, and CPM in particular is highly affordable. This is an important consideration as other immunotherapies, for example Ipilimumab, are prohibitively expensive. Subsequent studies are planned within the Godkin/Gallimore group to investigate the usefulness of CPM treatment in earlier stage CRC patients.

1.6 Concluding Remarks

Early CRC detection results in improved patient survival, however, this is a significant challenge. In addition, the detection of metastatic lesions in CRC patients can be difficult, particularly when they are in early development. It is reasonable to propose that reliable and time-efficient methods to detect CRC and associated metastasis may help to improve survival and could also represent a means of monitoring patients for disease relapse (see Question 1 overleaf).

In recent years, the advent of immunotherapy has revolutionised the way we treat cancer. Treatments such as checkpoint inhibition have proven that immunotherapy has the potential to induce sustained responses and long-term in survival in patients, albeit in a subset of patients and cancers. In CRC, patients with MSS or pMMR tumours do not respond to such therapies and highlight the need for improved treatment options.

TaCTiCC was recently completed within the Godkin/Gallimore group. Results have shown that T_{reg} depletion by CPM, and the induction of anti-tumour immune responses using 5T4 cancer vaccine TroVax, are able to convey significant survival benefits in a proportion of patients with advanced mCRC. These represent new and exciting treatment options that should be further explored.

I hypothesise that the identification of biomarkers to predict those patients likely to benefit from these novel treatments would enable better patient stratification (see Question 2 overleaf). Moreover, the identification of new antigen targets for CRC could enable the development of improved immunotherapies (see Question 3 overleaf).

1.7 Research Questions

This thesis addresses the following three questions:

1. Can a panel of plasma proteins identify earlier-stage and metastatic CRC patients from both each other, and from healthy donors?
    - It is hypothesised that reliable and time-efficient methods of CRC detection would improve survival. To address this question, a panel of plasma proteins were measured in patients with earlier-stage CRC, those with mCRC, and healthy donors, and their ability to identify patients was assessed. This investigation will be discussed in Chapter 3.

2. Can we identify biomarkers of immunotherapeutic response to both CPM and TroVax?
    - CPM and TroVax represent viable and effective treatment options for a proportion of patients with advanced mCRC. It is hypothesised that pre-treatment biomarkers to predict those patients most likely to benefit would allow us to target these patients more effectively. To address this, a panel of plasma proteins, immunological and serological measurements, and patient clinical and pathological data were assessed. This investigation is discussed in Chapter 4.

3. Can we identify novel antigen targets for CRC immunotherapy?
    - Although 5T4 vaccination with TroVax led to improved survival in TaCTiCC patients, this may not necessarily represent the optimum immunotherapy target. Targeting cancer antigens is difficult; they have frequent background expression on healthy tissue and may not be expressed on every tumour. To address this, T cell responses to 7 novel CRC tumour antigen candidates were assessed in healthy donors and CRC patients to assess their potential role as targets. This investigation is discussed in Chapter 5.

Chapter 2 Materials and Methods

Section (A) Luminex Analysis (Chapters 3 & 4)

2.1 Donors

Sections 2.1.1 - 2.1.4 relate to the donors, both CRC patients and healthy controls, for whom frozen plasma samples were used for Luminex experiments detailed in this thesis. Plasma samples were not subjected to multiple freeze-thaw cycles. The maximum time samples were frozen in liquid nitrogen was 6 years.

2.1.1 TaCTiCC Advanced Metastatic Colorectal Cancer Patients

Patients with advanced mCRC were previously recruited to the TaCTiCC clinical trial (n=52). Informed consent was obtained from all participants. This study was approved by The Gene Therapy Advisory Committee and the Cardiff and Vale University Healthy Board Committee. Findings from this study were published in 2017 (Scurr *et al*. 2017a; Scurr *et al.* 2017b). Each patient had inoperable stage IV disease with distant metastasis prior to trial enrolment. Throughout the trial, plasma samples were taken from patients, frozen, and stored in liquid nitrogen. The TaCTiCC treatment schedule, including when bloods were taken, is shown in Figure 2.1. These samples were used for Luminex experiments described within this thesis. Clinical and pathological features of these patients are shown in Table 2.1. In addition to these data, immunological and serological parameters were measured at several timepoints in these patients during TaCTiCC. These data were retrospectively investigated in this thesis and are summarised in Table 2.2. Normal ranges of these parameters are shown in Appendix Supplementary Table 3.

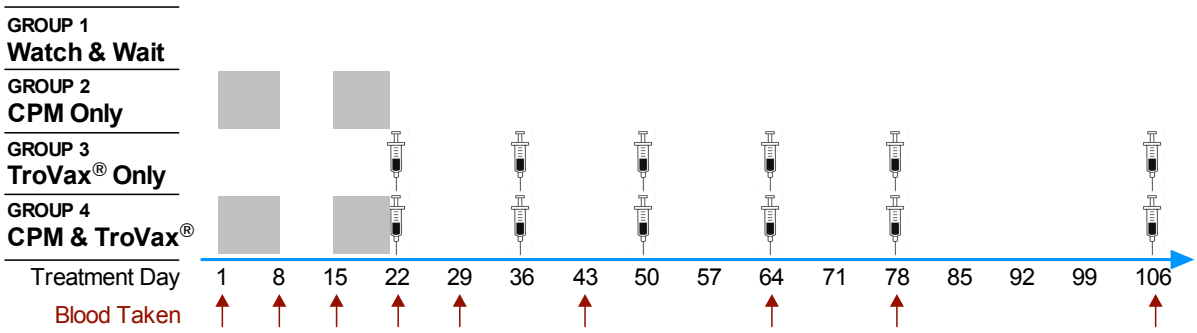


Figure 2.1 TaCTiCC treatment schedule.

TaCTiCC treatment schedule (Adapted from (Scurr *et al.* 2017a). In applicable groups, CPM treatment was given in 2 blocks; between TD1 and TD8, then between TD15 and TD22, and TroVax injections were subsequently given as indicated.

2.1.2 Non-Metastatic Colorectal Cancer Patients

In addition to the mCRC patients recruited to the TaCTiCC clinical trial, a cohort of patients with non-metastatic CRC were included in Luminex analysis (n=14). These patients were previously recruited to studies within the Godkin/Gallimore group and had available frozen plasma samples stored in liquid nitrogen. Samples were collected with local research ethics committee approval, and informed consent was obtained for each patient. These patients and frozen plasma samples represent the non-metastatic CRC cohort used for Luminex experiments described within this thesis. Clinical and pathological features of these patients are shown in Table 2.3.

TaCTiCC Patient Characteristics Luminex Experiments (n=52)		Group 1 (Control) n=8	Group 2 (CPM) n=9	Group 3 (TroVax) n=17	Group 4 (Dual) n=18
Sex	Female	2 (25%)	5 (56%)	3 (18%)	4 (22%)
(%)	Male	6 (75%)	4 (44%)	14 (82%)	14 (78%)
Age	Range	47-75	56-72	35-81	41-81
	Mean	63	65	63	66
	Median	63	65	64	67
Tumour Location	Right	1 (12.5%)	4 (44%)	3 (18%)	1 (6%)
(%)	Left	4 (50%)	3 (33%)	8 (47%)	6 (33%)
	Sigmoid/Rectum	3 (37.5)	2 (22%)	6 (35%)	8 (44%)
	Unknown	0 (0%)	0 (0%)	0 (%)	3 (17%)
TNM Stage	IV	8 (100%)	9 (100%)	17 (100%)	18 (100%)
Dukes' Stage	D	8 (100%)	9 (100%)	17 (100%)	18 (100%)
Site of Metastases	Liver	6 (75%)	5 (56%)	8 (47%)	14 (78%)
	Lung	4 (50%)	4 (44%)	9 (53%)	7 (39%)
	Peritoneum	5 (63%)	4 (44%)	3 (18%)	7 (39%)

Table 2.1 Advanced mCRC TaCTiCC patient characteristics used for Luminex experiments.

This table outlines the characteristics of patients with advanced mCRC enrolled on the TaCTiCC clinical trial (n=52). Patients are split by trial group; Group 1 received no treatment, Group 2 received CPM treatment, Group 3 received TroVax treatment, and Group 4 received dual CPM/TroVax treatment. These patients represent the mCRC patient group used to perform Luminex experiments, the results of which are presented in Chapters 3 and 4.

Factor	Units
5T4 antibody response	Relative units (RU)
5T4 cultured T cell response	spot forming cells/10^5 PBMC/epitope
Basophils	$x10^9$/L
Bilirubin	µmol/L
$CD3^+CD4^+$ cells	per µl whole blood
$CD3^+CD8^+$ cells	per µl whole blood
Eosinophils	$x10^9$/L
Glucose	mmol/L
Haematocrit (Haem)	L/L (% of blood)
Haemoglobin (Hb/Hgb)	g/dL
Lymphocytes	$x10^9$/L
MCH (Mean corpuscular haemoglobin)	pg
MCV (Mean corpuscular volume)	fL
Monocytes	$x10^9$/L
MVA antibody response	Relative units (RU)
Neutrophil: Lymphocyte Ratio (NLR)	Ratio
Neutrophils	$x10^9$/L
Platelets	$x10^9$/L
Red blood cell count (RBC)	$x10^{12}$/L
Alanine aminotransferase (ALT)	U/L
T_{reg} number	per µl whole blood
T_{reg} proportion	($CD4^+Foxp3^+$/Total $CD4^+$) x100
White blood cell count (WBC)	$x10^9$/L

Table 2.2 Immunological/serological parameters measured during TaCTiCC, investigated retrospectively in this thesis.

Non-Metastatic CRC Patient Characteristics Luminex Experiments (n=14)		
Sex (%)	Female	6 (43%)
	Male	8 (57%)
Age (mean)	Range	52-82
	Mean	66
	Median	64
Tumour Location (%)	Right	5 (36%)
	Left	0 (0%)
	Sigmoid/Rectum	6 (43%)
	Transverse	2 (14%)
	Unknown	1 (7%)
TNM Stage	Pre-TNM	1 (7%)
	T1	1 (7%)
	T2	0 (0%)
	T3	9 (64%)
	T4a	1 (7%)
	T4b	2 (14%)
Lymph Node Spread	N0	8 (57%)
	N1	5 (36%)
	N2	1 (7%)
Dukes' Stage	Pre-A	1 (7%)
	A	1 (7%)
	B	6 (43%)
	C1	6 (43%)
	C2	0 (0%)

Table 2.3 Luminex non-metastatic CRC patient characteristics.

This table outlines the characteristics of patients with non-metastatic CRC used to perform Luminex experiments, the results of which are presented in Chapters 3 and 4. The pre-TNM patient had tubulovillous adenoma.

2.1.3 Histopathological Staging/Tumour Grading

TaCTiCC patients were assessed for trial eligibility at either South West Wales Cancer Centre, Swansea, or Velindre National Health Service Trust, Cardiff. All patients had confirmed non-operable stage IV mCRC prior to enrolment. Histopathological staging and tumour grading of the non-metastatic CRC group was confirmed by consultant pathologists within University Hospital Wales using TNM 8th Edition. TNM staging was converted to Dukes' staging for further analysis. Although Dukes' is a historical staging system, it provides a simplistic method to meaningfully split small patient groups. TNM staging, Dukes' staging, and TNM to Dukes' conversion are described below:

TNM Classification

pT Stage – Relating to size and extent of primary tumour

T0: No evidence of primary tumour

Tis: Intramucosal carcinoma, without extension to the submucosa

T1: Tumour invades the submucosa, but does not invade the muscularis propria

T2: Tumour invades the muscularis propria

T3: Tumour invades through the muscularis propria, and extends into the serosa

T4: Tumour has grown into adjacent tissues

T4a: Tumour penetrates visceral peritoneum

T4b: Tumour directly invades other organs/tissues

pN Stage – Relating to regional lymph node involvement/metastasis

N0: No involvement of regional lymph nodes

N1: Involvement of 1-3 regional lymph nodes

N1a: Involvement of 1 regional lymph node

N1b: Involvement of 2-3 regional lymph nodes

N1c: No regional lymph nodes, however there are tumour deposits in lymph drainage area of the primary tumour

N2: Involvement of 4+ regional lymph nodes

N2a: Involvement of 4-6 regional lymph nodes

N2b: Involvement of 7+ regional lymph nodes

pM Stage – Relating to distant metastasis

M0: No evidence of distant metastasis

M1: Distant metastasis

M1a: Distant metastasis in 1 organ/site. No peritoneal metastasis

M1b: Distant metastasis in 2+ organs/sites. No peritoneal metastasis

M1c: Peritoneal metastasis alone, or in addition to distant metastasis

N.B. T, N, or MX – Feature cannot be assessed. M0 not assigned by pathologist.

Dukes' Classification

Dukes' A: Tumour invasion limited to the bowel wall

≈ Stage 1

Dukes' B: Tumour invades through the bowel wall and no lymph node involvement

≈ Stage II

Dukes' C: Regional lymph node involvement/metastasis

≈ Stage III

Dukes' C1: Regional lymph node involvement/metastasis

Dukes C2: Lymph node involvement reaches apical lymph node

Dukes' D: Presence of distant metastasis

≈ Stage IV

TNM/Dukes' Conversion

Dukes' A: T1/T2 primary tumours without lymph node or distant metastasis

(pT1/2, N0, M0)

Dukes' B: T3/T4 primary tumours without lymph node or distant metastasis

(pT3/4, N0, M0)

Dukes' C: Any T stage with lymph node involvement and without distant metastasis

(pT1-4, N1, M0) or (pT1-4, N2, M0)

Dukes' D: Any T and N stage, with distant metastasis

(pT1-4, N1/2, M1)

2.1.4 Healthy Donors

In addition to the aforementioned mCRC and non-metastatic patient cohorts, a healthy donor group (n=39) was also investigated in the context of the Luminex experiments detailed in Chapters 3 and 4. Most donors were specifically recruited for this study; however, a small number were consented to previous studies within the Godkin/Gallimore group. Samples were collected with local research ethics committee approval, and informed consent was obtained for each donor. These donors and frozen plasma samples represent the healthy donor cohort used for Luminex experiments described within this thesis and are detailed in Table 2.4.

Healthy Donor Characteristics Luminex Experiments (n=39)		
Sex (%)	Female	19 (49%)
	Male	20 (51%)
Age	Total mean	47
Under 30	Range 23-29	11 (28%)
	Median = 25	
30-60	Range 34-59	15 (38%)
	Median = 46	
Over 60	Range 61-75	12(31%)
	Median = 70	
Not recorded		1 (3%)

Table 2.4 Luminex healthy donor characteristics.

This table outlines the characteristics of healthy donors used to perform Luminex experiments, the results of which are presented in Chapters 3 and 4.

2.2 Isolation of Plasma from Whole Blood

Approximately 10ml whole blood was drawn from donors into a heparinised vacutainer. To ensure sterility, subsequent steps were performed inside a Class II Laminar Flow Hood (BioQuell, Microflow – Class II). 20ml of sterile Lymphoprep (Alere International Limited, Product Code 111454) was added into a sterile 50ml falcon tube. Using a sterile 10ml stripette, whole blood was carefully layered on top of the Lymphoprep and centrifuged at room temperature at 2000 rpm for 20 minutes. Using a sterile Pasteur pipette, the plasma fraction was collected into 1ml aliquots. Aliquots were put in a Nalgene Freezing Container (Mr. Frosty Freezing Container, Thermo Fisher Scientific), and placed at -80°C prior to transfer to liquid nitrogen for long-term storage.

2.3 Luminex

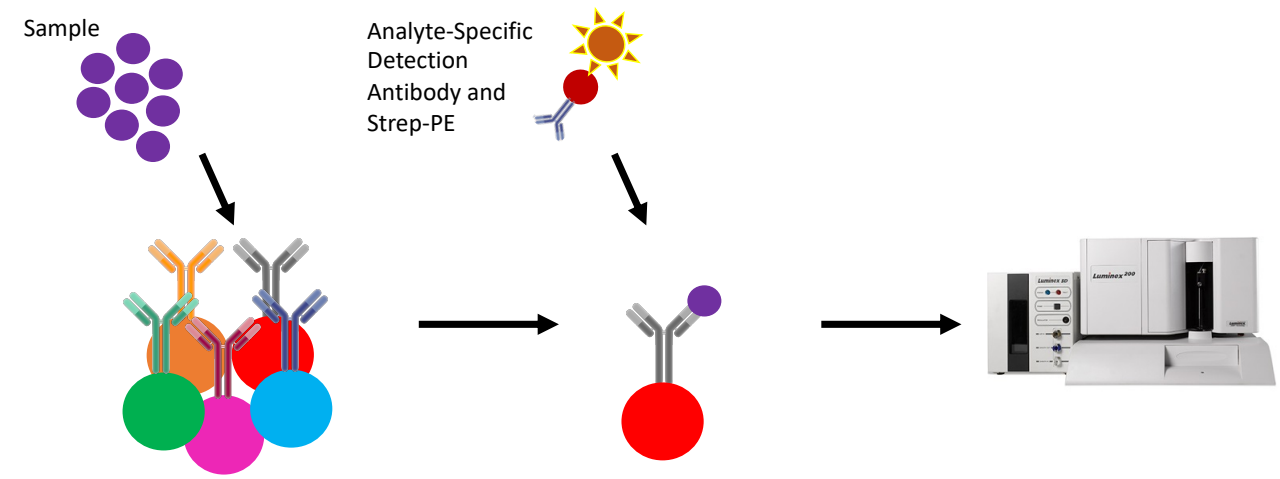
2.3.1 Luminex Principle

Luminex is a simple and time-effective bead-based assay which allows the simultaneous detection and quantification of multiple analytes within the same sample. This is summarised in Figure 2.2. Briefly:

1. Sample, for example plasma, is added to a mixture of beads pre-coated with analyte-specific capture antibodies. These beads bind to the analyte of interest. Beads for each analyte are spectrally distinct to allow for classification, thus the ability to multiplex.

2. Analyte-specific biotinylated detection antibodies are added, followed by Phycoerythrin (PE)-conjugated streptavidin, which binds to the biotinylated detection antibody.

3. Beads are run through Luminex machine. One laser classifies the bead; therefore, the analyte being assessed. A second laser quantifies the magnitude of PE signal; therefore, the amount of the analyte bound.



STEP 1
Sample added to a mixture of colour-coded beads, precoated with analyte-specific capture antibodies.

STEP 2
Biotinylated detection antibodies specific for the analytes are added. Strep-PE binds these.

STEP 3
Analysed on Luminex Machine. One laser classifies the bead, second laser determines magnitude of PE-signal.

Figure 2.2 Diagrammatic overview of Luminex technique.

2.3.2 Panel Design

In order to measure the levels of proteins within the plasma of the donors described in Sections 2.1.1, 2.1.3, and 2.1.4, Luminex Multiplex was performed. The final panel of plasma proteins investigated by Luminex (n=31) was determined based on both previous association of the proteins with cancer and/or immunity, and through a series of preliminary experiments performed on a cross-section of mCRC TaCTiCC patients. These experiments were used to refine the panel and are not the focus of this thesis. An overview of the final panel proteins, and their relationship to cancer/immunity is shown in Appendix Supplementary Table 2. These proteins were configured into 4 separate assays; 1-plex, 4-plex, 5-plex, and 21-plex, based on both required plasma dilution factor, and assay provider. This is detailed in Table 2.5.

Analyte	Dilution Factor	Kit Provider
APOA1 *Single-Plex Assay*	1:4000	R&D Systems, Bio-Techne Human Magnetic Luminex Assays Cat. Number: LXSAHM-01/1472939 Lot Number: L121535
CCL17 **MMP-8** **MMP-10** **MMP-12** **Tenascin-C** *5-Plex Assay*	1:2	R&D Systems, Bio-Techne Human Magnetic Luminex Assays Cat Number: LXSAHM-05/1472940 Lot Number: L121536
MMP-2 **MMP-3** **MMP-9** **TIMP-1** *4-Plex Assay*	1:50	Thermo Fisher Scientific ProcartaPlex Mix & Match (Magnetic) Cat. Number: PPX-04-MXDJXJV Lot Number: 157837000

Table 2.5 Overview of plasma proteins assessed by Luminex (continued overleaf).

Each of the proteins measured in the plasma of patients/donors is shown. Proteins were split into 4 assays, based on required dilution and provider availability as shown.

Analyte	Dilution Factor	Kit Provider
BDNF	1:2	Thermo Fisher Scientific
BTLA		ProcartaPlex Mix & Match (Magnetic)
CD27		Cat. Number: PPX-21-MXCE3YX
Eotaxin		Lot Number: 159645000
HGF		
IDO		
IL-2		
IL-15		
IL-21		
IL-22		
IL-27		
IP-10		
MMP-1		
MMP-7		
MMP-13		
PIGF-1		
RANTES		
SDF-1α		
TIM-3		
VEGF-A		
4-1BBL		
21-Plex Assay		

Table 2.5 (continued) Overview of plasma proteins assessed by Luminex.
Each of the proteins measured in the plasma of patients/donors is shown. Proteins were split into 4 assays, based on required dilution and provider availability as shown.

2.3.3 Luminex Protocol

Luminex was performed as per the standard operating procedure (SOP) provided with each kit. This was performed on frozen plasma samples from patients and healthy donors described in Section 2.1.1, 2.1.2, and 2.1.4. Due to cost, this was performed once for each sample. Exact steps were slightly altered between the two kit providers; however, the general principles remained the same. A generalised protocol is summarised below:

1. Frozen plasma samples were thawed on ice, vortexed, and centrifuged for 5 minutes at 10,000 g. Assay-specific sample dilutions were prepared, as detailed in Table 2.5, using kit-specific diluent.
2. Antigen standards were reconstituted, and serial dilutions prepared, as per SOP.
3. Magnetic beads were resuspended and added to each well as directed by kit SOP.
4. Plasma sample/antigen standards were added to respective wells, as per kit SOP. Plasma samples were performed in single, and antigen standards were performed in duplicate.
5. Plate covered with adhesive plate cover and incubated on horizontal plate shaker at 800 rpm for 60-120 minutes, as directed by SOP.
6. Plate inserted into hand-held magnetic plate washer and washed x3 with kit-specific wash buffer.
7. Biotin detection antibody prepared and added to wells, as directed by kit SOP. Plate sealed with adhesive plate cover and incubated on horizontal plate shaker at 800 rpm for 30-60 minutes, as per kit SOP.
8. Plate washed as per Step 6.
9. Streptavidin solution prepared and added to wells as per kit SOP. Plate sealed and incubated on horizontal plate shaker at 800 rpm for 30 minutes.
10. Plate washed as per Step 6.
11. Magnetic beads resuspended in kit-specific reading buffer, plate sealed with adhesive plate cover, and incubated on horizontal plate shaker at 800 rpm for 2-5 minutes.
12. Plate run on Luminex 200 machine within 90 minutes. Machine settings as per kit SOP.

Quantification of each analyte, accounting for dilution factor, was obtained. This data was generated by the Luminex software, using the standard curve for each analyte. An example of the output/standard curve generated is shown in Figure 2.3 and 2.4. If the level of plasma protein was below the lowest point on the standard curve, the analyte was considered undetectable, and therefore assigned a value of 0 pg/ml. Similarly, if the plasma protein was above the highest point on the standard curve, it was assigned the highest value on the standard curve.

9	Analyte	Type	Well	Description	FI	FI - Bkgd	Std Dev	%CV	Obs Conc	Exp Conc	(Obs/Exp) * 100	Dilution
10	TIMP-1 (26)	B	A8,B8		105	105	2.83	2.69				1
11	TIMP-1 (26)	S1	A1,B1		4807	**4702**	158.39	3.3	151057.96	150000	**101**	1
12	TIMP-1 (26)	S2	A2,B2		3571.8	**3466.8**	176.42	4.94	37366.57	37500	**100**	1
13	TIMP-1 (26)	S3	A3,B3		1515.3	**1410.3**	37.12	2.45	9435.53	9375	**101**	1
14	TIMP-1 (26)	S4	A4,B4		529	**424**	4.24	0.8	2321.73	2343.75	**99**	1
15	TIMP-1 (26)	S5	A5,B5		221.3	**116.3**	10.96	4.95	584.57	585.94	**100**	1
16	TIMP-1 (26)	S6	A6,B6		128.5	**23.5**	4.95	3.85	153.04	146.48	**104**	1
17	TIMP-1 (26)	S7	A7,B7		97.5	**-7.5**	10.61	10.88	33.99	**36.62**	**93**	1
18	TIMP-1 (26)	X1	A9	120 TD1	1204	1099	0	0	348027.3			50
19	TIMP-1 (26)	X2	A10	120 TD22	1158	1053	0	0	330679.8			50
20	TIMP-1 (26)	X3	A11	120 TD43	836.5	731.5	0	0	215715.71			50

Figure 2.3 Example of data output from Luminex experiment.

This is a representative example of the data generated for Luminex experiments for TIMP-1. Row 10 is data generated for blank wells as an assessment of background measurements. Rows 11-17 are data generated relating to the standard curve, and rows 18-20 are data relating to measured plasma samples for patient 120. Column FI is fluorescence intensity, FI-Background is fluorescence intensity minus background intensity from Row 10. % CV is the variance between the duplicate standard curve values obtained. Observed and expected concentrations are shown for standard curve, and observed concentration is shown for assessed plasma samples. Dilution factor is shown and is automatically accounted for.

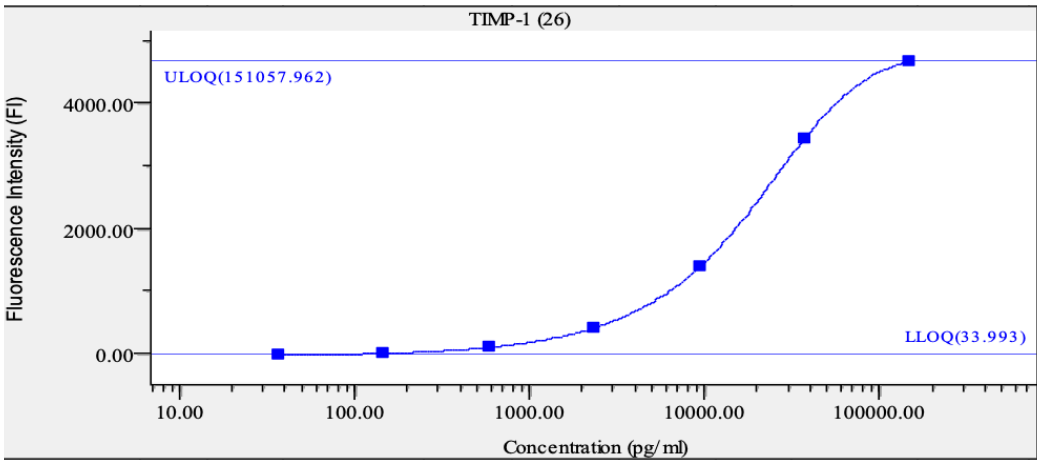


Figure 2.4 Standard curve for TIMP-1.

This standard curve was generated for the assay shown in Figure 2.3, from the data shown in rows 11-17.

2.4 Statistical and Graphical Analysis

2.4.1 Comparison of Levels of Plasma Proteins

The normality in distribution of the plasma protein measurements obtained for healthy donors, non-metastatic CRC patients, and CRC patients was tested by Shapiro-Wilk (Chapter 3). Shapiro-Wilk is a test of normality of data; it tests the null hypothesis that ***sample x*** is derived from a normally distributed population. Where data were normally distributed, differences between groups were then compared by Welch's t-test and ANOVA. Where data were not normally distributed, differences between groups were then compared by Mann-Whitney U-test/Spearman's rank correlation coefficient. Results were considered to be statistically significant if $p<0.05$. Asterix representation of significance is as described in Table 2.6. This methodology was also used to compare plasma protein/immunological parameter differences between mCRC TaCTiCC patients who responded/did not respond to CPM and/or TroVax treatment, and to compare these parameters between patients split by clinical parameters (Chapter 4). These statistical/graphical analyses were performed using GraphPad Prism.

p value	Asterix Representation
> 0.05	Not significant (ns)
≤ 0.05	(*)
≤ 0.01	(**)
≤ 0.001	(***)
≤ 0.0001	(****)

Table 2.6 Asterix representation of levels of statistical significance used within this thesis.

2.4.2 Cluster Analysis of Plasma Proteins

To investigate how the combination of several plasma proteins can distinguish between healthy donors, mCRC patients, and CRC patients without metastasis, hierarchical cluster analysis was performed. This was performed in R using the hclust() function and Ward's minimum variance method (ward.D2). Using Ward's method, each observation (patient/donor) is initially assigned to their own cluster. At each stage, a new cluster is formed which minimises the total within-cluster variance, until every observation is combined into one cluster containing all observations. At each stage, every possible combination of clusters is considered before making an assignment. Subsequent to this, the cutree() function was used to split the output from hclust() into two clusters. The number of CRC patients and healthy donors in each cluster was then calculated and used as a measure of sensitivity/specificity. Heatmaps to reflect these analyses were generated using the pheatmap() function.

2.4.3 Receiver-Operating Characteristic (ROC) Curve Analysis

To evaluate each individual plasma protein as a diagnostic test, ROC curve analysis was performed using GraphPad Prism. ROC curves are generated by plotting sensitivity (the number of correctly identified individuals with disease) against specificity (the number of correctly identified individuals without disease) at various thresholds. Youden's Index (Youden's J statistic) was used to select the optimum cut-off value, in pg/ml, for each plasma protein. If the test were perfect, Youden's Index would be 1. It is calculated using the below formula:

$$J = \text{sensitivity} + \text{specificity} - 1$$

An associated area under the curve (AUC) was computed during ROC curve analysis and was used as an assessment of overall diagnostic ability. For reference, a test with no diagnostic capacity would have an AUC of 0.5, and a "perfect" test would have an AUC of 1. In addition to this, a p-value associated with the ROC curve was generated, testing the null hypothesis that the AUC = 0.50. An overall assessment of diagnostic accuracy was made based on the area under the ROC curve, as described in Table 2.7. This methodology was also used to produce ROC curves from the logistic predictor scores derived from logistic regression modelling (see Section 2.4.5).

Area Under Curve (AUC)	Diagnostic Accuracy
0.9-1.0	Excellent
0.8-0.9	Very good
0.7-0.8	Good
0.6-0.7	Sufficient
0.5-0.6	Bad
< 0.5	Not useful

Table 2.7 Relationship between area under ROC curve and diagnostic accuracy (adapted from (Šimundić 2009).

2.4.4 Correlation of Plasma Proteins

Correlation of plasma proteins was assessed in GraphPad Prism using Spearman's Rank correlation coefficient, as data were not normally distributed. Correlations were considered of potential interest if $p<0.05$ and $r \geq +/- 0.3$. The magnitude of correlation was interpreted as per Table 2.8. This methodology was also used to correlate the levels of potential treatment biomarkers with age (Chapter 4).

Size of Correlation (Spearman r value)	Interpretation
+/- 0.90 to 1.00	Very high positive/negative correlation
+/- 0.70 to 0.90	High positive/negative correlation
+/- 0.50 to 0.70	Moderate positive/negative correlation
+/- 0.30 to 0.50	Low positive/negative correlation
+/- 0.00 to 0.30	Negligible Correlation

Table 2.8 Interpreting the size of a correlation coefficient (Mukaka 2012).

2.4.5 Data Modelling

To assess the diagnostic capacity of combining multiple plasma proteins for distinguishing advanced mCRC and earlier-stage CRC patients from one another, and from healthy donors (Chapter 3), logistic regression analysis was performed. This was performed in R using the glm() function. Logistic regression allows statistical modelling of a binary dependent variable, e.g. presence of absence of advanced mCRC. Several logistic regression models were built using backwards selection. Backwards selection begins by including all variables of interest in the model, and at each step removes the least statistically significant variable, until all remaining variables are significant. Results of each model were exported, and the logistic predictor values obtained were used to perform ROC curve analysis, as per Section 2.4.3. This methodology was also used to model the prediction of advanced mCRC patient response to CPM/TroVax treatment using single and multiple pre-treatment immunological/plasma protein biomarkers (Chapter 4).

The accuracy of the models from Chapter 3 were tested by cross-validation. To perform this, 75% of the total data (patients and controls) was randomly assigned to a training dataset, using the sample.split() function in R. This function splits the data into two sets, based on a pre-defined ratio, while preserving the relative ratios of different labels i.e. number of patients with cancer versus those without. These data were used to train the logistic regression model. This model was then tested on the remaining 25% of data, and the accuracy of the model was assessed based on the number of correctly identified patients and controls by using a confusion matrix.

2.4.6 Association of Pre-Treatment Biomarkers with Survival

Potential biomarkers of interest for predicting response to CPM/TroVax immunotherapy were investigated for their association with survival in advanced mCRC patients from TaCTiCC. This was performed for both overall and progression-free survival by log-rank test and was displayed by Kaplan-Meier plot. Analysis was performed using R and graphical output using GraphPad Prism.

2.4.7 Changes in Levels of Biomarkers During Treatment

To investigate changes in levels of immunological/plasma protein biomarkers across treatment, GraphPad Prism was used to graph levels across time. Differences between timepoints were compared by paired t-test or Wilcoxon signed-rank test, depending on normality of data distribution.

Section (B) Novel Tumour Antigen Candidate Analysis (Chapter 5)

2.5 Donors

Sections 2.5.1 and 2.5.2 relate to the patients and healthy donors used to investigate the immunogenicity of novel tumour antigen candidates.

2.5.1 Colorectal Cancer/Other Patients

Patients undergoing clinical investigation of the colon were recruited from University Hospital Wales. Whole blood samples were collected with local research ethics committee approval, and informed consent was obtained for each patient. Patient follow-up was used to ascertain disease status, and where appropriate TNM/Dukes' staging was obtained, as per Section 2.1.3. CRC patients are summarised in Table 2.8. Diseases of the colon were noted in patients without CRC, and these patients are summarised in Table 2.10. In addition to this cohort, frozen PBMC samples from a cohort of TaCTiCC patients receiving CPM treatment were investigated. These patients are summarised in Table 2.9.

2.5.2 Healthy Donors

Healthy donors were recruited from Cardiff University School of Medicine, Henry Wellcome Building. Informed consent was obtained from each participant. Whole blood samples were collected with local research ethics committee approval. These donors are summarized in Tables 2.11-2.13.

Patient ID	*Age (74)*	*Gender*	*Tumour Location*	*TNM Stage*			*Dukes' Stage*
				T	N	M	
****CRW-D7-11***	***80***	***M***	***Caecum***	***4b***	***0***	***X***	***B***
****CRW-EC-1***	***78***	***M***	***Rectum***	***4a***	***0***	***0***	***B***
****CRW-EC-2***	***72***	***M***	***Caecum***	***4a***	***2a***	***X***	***C***
CRW-EC-3	74	M	Transverse Colon	1	0	0	A
****CRW-EC-4***	***71***	***F***	***Ascending Colon***	***4a***	***2a***	***1***	***D***
CRW-EC-7	74	F	Ascending Colon	2	1a	0	C
CRW-EC-10	67	F	Rectum	2	1a	0	C
CRW-EC-11	74	M	Caecum	2	1a	0	C

Table 2.9 CRC patients in whom the immunogenicity of novel tumour antigen candidates was investigated.

This table represents all CRC patients in whom the immunogenicity of the novel tumour antigen candidates was investigated by primary T cell culture (n=8). Those patients indicated in bold are those patients in whom antigen immunogenicity was also investigated *ex vivo* (n=4). TNM and Dukes' stage shown. Mean group age of all patients is 74 and mean age of patients analysed *ex vivo* is 75.

Patient ID (TaCTiCC)	*Age (59)*	*Gender*	*Tumour Location*	*CPM Responder*
101	57	M	Rectum	YES
102	57	F	Right	YES
109	67	M	Rectum	YES
113	54	F	Undefined	YES
116	62	M	Right	NO

Table 2.10 TaCTiCC mCRC patients receiving CPM treatment in whom the immunogenicity of novel tumour antigen candidates was investigated.
This table represents the TaCTiCC patients receiving CPM treatment in whom the immunogenicity of the novel tumour antigen candidates was investigated by primary T cell culture (n=5) using frozen PBMC samples. All patients had inoperable Stage IV/Dukes' D disease. Mean group age is 59.

Patient ID	*Age (59)*	*Gender*	*Condition*
***CRW-EC-5**	***31***	***F***	***Previous polyp cancer (has current polyps)***
CRW-EC-6	69	F	Previous polyp cancer (has current polyps)
CRW-EC-8	57	M	Long-term ulcerative colitis
CRW-EC-12	67	M	Recurrent benign polyps (has current polyps)
CRW-EC-13	71	F	Recurrent benign polyps (has current polyps)

Table 2.11 Other patients in whom the immunogenicity of novel tumour antigen candidates was investigated.
This table represents all other patients in whom the immunogenicity of the novel tumour antigen candidates was investigated by primary T cell culture (n=5). Those patients indicated in bold are those patients in whom immunogenicity was also investigated *ex vivo* (n=1). As these patients were recruited upon attendance of gastroenterology pre-clinical appointments, known related medical conditions are shown. Mean group age of all patients is 59.

Patient ID	*Age (27)*	*Gender*
HD1	***26***	***F***
HD2	25	M
HD3	36	F
HD5	27	M
HD6	25	M
HD8	25	M

Table 2.12 Healthy donors in whom the immunogenicity of control antigens was investigated *ex vivo.*

This table represents healthy donors used for the *ex vivo* investigation of response to control antigens (n=6). This was used to validate and optimise the use of ImmunoSpot techniques. The donor in bold is the individual in whom responses to control antigens across time were assessed. Mean group age of all patients is 27.

Patient ID	*Age (30)*	*Gender*
HD1	26	F
HD2	25	M
HD3	36	F
HD12	29	M
HD16	25	F
HD18	35	F
HD19	23	M
HD25	36	F
HD28	26	M
HD29	48	F
HD34	26	M

Table 2.13 Healthy donors in whom the immunogenicity of novel tumour antigen candidates was investigated *ex vivo.*

This table represents the healthy donors in whom the immunogenicity of novel tumour antigen candidates was investigated *ex vivo* (n=11). Mean group age is 30.

Patient ID	*Age (32)*	*Gender*
HD1	26	F
HD13	30	M
HD16	25	F
HD18	35	F
HD21	28	F
HD25	36	F
HD28	26	M
HD34	28	M
CRW-EC-9	55	M

Table 2.14 Healthy donors in whom the immunogenicity of novel tumour antigen candidates was investigated by primary T cell culture.

This table represents the healthy donors in whom the immunogenicity of novel tumour antigen candidates was investigated by primary T cell culture (n=9). Mean group age is 32.

2.6 Lymphocyte Isolation

2.6.1 Isolating PBMC from Whole Blood

Between 30 and 50ml of whole blood was obtained from each donor into heparinised vacutainers. Isolation of PBMC was performed inside a Class II Laminar Flow Hood (BioQuell, Microflow – Class II) to ensure sterility. To isolate PBMC, up to 20ml whole blood was layered on top of 20ml Lymphoprep (Alere International Limited, Product Code 111454) and centrifuged at 2000 rpm (with no brake) for 20 minutes at room temperature. R+ media was prepared by adding Pen/Strep and L-glutamine (Gibco, Cat. Number 10378-016) and sodium pyruvate (Gibco, Cat. Number 11360-039) to 500ml RPMI-1640 (Gibco, Cat. Number E15-041) at final concentrations of x1 and 1mM respectively. R+ was pre-warmed in a water bath. Using a Pasteur pipette, the PBMC layer was extracted and added to R+ to a final volume of 40ml and centrifuged at 2000 rpm for 10 minutes at room temperature. Supernatant was poured away and pellet resuspended in 5ml of x1 red blood cell (RBC) lysis buffer and incubated at room temperature for 5 minutes. 20ml R+ was added to inactivate the RBC lysis buffer and passed through a 70μm cell strainer into a fresh tube. This was centrifuged at 1600 rpm for 5 minutes at room temperature. Supernatant was poured away and pellet resuspended in 10ml R+ media. At this stage, PBMC were enumerated. Prior to setting up *ex vivo* FluoroSpot, or cultured T cell lines, cells were centrifuged again at 1600 rpm for 5 minutes at room temperature and resuspended in the required volumes of pre-warmed CTL test plus media. CTL test plus media (CTL, Cat. Number CTLTP-005) was prepared by adding Pen/Step and L-glutamine, and sodium pyruvate to CTL test plus medium, at final concentrations of x1 and 1mM respectively.

2.6.2 PBMC Enumeration

PBMC were enumerated by flow cytometry, using the Novocyte 3000 machine (ACEA). 50μl of media containing the isolated, resuspended PBMC, was added to a 5ml FACS tube (BD). 0.5μl of propidium iodide (Sigma-Aldrich, Cat. Number P4864-10ML) was added, mixed, and left for 2 minutes at room temperature. 20μl of sample was run through the Novocyte at medium speed, and gating adjusted accordingly to identify live PBMC. This is summarised in Figure 2.5. Absolute counts were used to enumerate the total number of live PBMC, i.e. if absolute count was 1000/μl, and there was 10ml of PBMC sample, total count was $1x10^7$ PBMC.

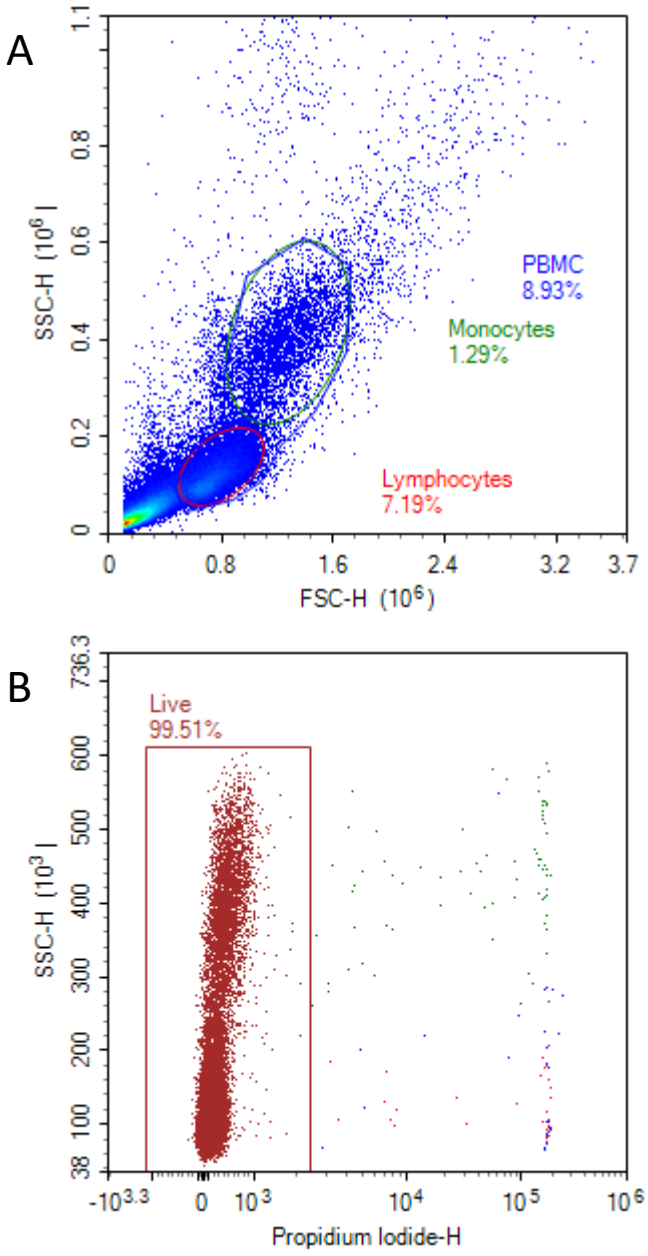


Figure 2.5 Representative example of gating strategy for live PBMC enumeration.

0.5 µl of Propidium Iodide (PI) added to 50µl of resuspended PBMC sample. 20µ of sample was run through the Novocyte. PBMC subsets were gated based on FSC/SSC, and whole PBMC taken as the overall population of these subsets (A). Whole PBMC were then gated by live cells, using PI (B).

2.6.3 Freezing/Thawing PBMC

To freeze any excess PBMC, they were resuspended at a concentration of approximately $5x10^6$ in freezing media. Freezing media was prepared by combining 90% Fetal Calf Serum (FCS) with 10% DMSO. 1ml aliquots were placed inside of a Mr. Frosty container and placed into -80°C freezer, prior to transfer to liquid nitrogen for long-term storage.

To thaw frozen TaCTiCC patient PBMC samples, vials were obtained from the liquid nitrogen, and placed on ice. Aliquots were placed into a 37°C water bath until they were nearly thawed. Using a P1000, cells were drop-wise added to 10ml pre-warmed R+ media. Cells were washed by centrifuging at 1600 rpm for 5 minutes at room temperature and resuspended in 10ml R+ prior to PBMC enumeration.

2.7 Antigens

Purified protein derivative of tuberculin (PPD) and tetanus toxoid (TT) were purchased from Statens Serum Institute, and phytohemagglutinin (PHA) was purchased from Sigma-Aldrich. Haemagglutinin (HA) strain X31 was a gift from Dr John Skehel, (National Institute of Medical Research, London, United Kingdom). Each of these control antigens were whole protein and were used at a final concentration of 1-10μg/ml.

20mer peptides overlapping by 10 amino acids, covering the entire protein sequence of each novel tumour antigen candidate, were synthesised to >95% purity by GL Biochem, Shanghai, China. These peptides were divided into pools as shown in Supplementary Tables 1.1-1.7. Stock concentration of each peptide was 50mg/ml. Each peptide pool was used at a final concentration of 1-5μg/ml per peptide. 20mer peptides overlapping by 10 amino acids covering the entire protein sequence of 5T4 were also used. These were split into two peptide pools, as shown in Supplementary Table 1.8. These were also used at a final concentration of 1-5μg/ml per peptide.

2.8 Primary T Cell Culture

All steps to establish primary T cell cultures were performed inside a Class II Laminar Flow Hood in sterile conditions. PBMC were isolated as described and resuspended in CTL test plus at $2x10^6$ per ml. CTL test plus media was prepared as described, with the addition of Pen/Strep and L-glutamine, and sodium pyruvate, at final concentrations of x1 and 1mM respectively. 100µl of cells (200,000 cells) were added per well to a 96-well plate. Lines were established in duplicate in 96-well round-bottom plates to each of the peptide pools for the novel tumour antigens, and to controls antigens Ha, PPD, and TT. Response to PHA was used as a positive control for subsequent Immunospot assays. The number of lines set up was dependent on the number of PBMC isolated for each patient. Plates were kept in sterile conditions in a 37oC incubator with 5% CO2, and lines were grown for 14 days. Cells were supplemented with 100µl of fresh CTL test plus media, with 40 units/ml of IL-2, on days 3, 7, and 10, before analysing by FluoroSpot on day 14. Final volume on day 14 was 200 µl per well.

2.9 IFNγ/Granzyme B FluoroSpot

IFNγ/Granzyme B FluoroSpot kits and FluoroSpot PVDF plates were purchased from MabTech (Product Codes FS-0110-10 and 3654-FL-10). FluoroSpot assays were performed inside a Class II Laminar Flow Hood in sterile conditions. Plate membranes were wet with 30µl of 35% ethanol for 1 minute then washed x5 with 150µl/well sterile PBS (Thermo Fisher, Product Code 10010056). Coating antibodies 1-D1K and GB10 were prepared to 15µg/ml in PBS, and 50µl added per well. Plate was sealed with parafilm and incubated in a fridge at 4oC overnight. Coating antibodies were removed from wells by flicking, and wells were washed x5 with 150µl/well sterile PBS. 50µ per well of CTL test plus media was added and incubated in 37oC incubator with 5% CO2 for 30 minutes. At this stage PBMC were isolated and enumerated as described and resuspended at the required concentration in CTL test plus media (200-250,000 cells per 100µl for *ex vivo* assays, and 50-100,000 cells per 100µl for cultured assays). PBMC were added to corresponding wells on top of 50µl media used for blocking and stimulated with peptide at required concentration. Plates were incubated in 37oC incubator with 5% CO2 for 24 hours. Cells were removed by flicking and wells were washed x5 with 150µl/well sterile PBS. Detection antibodies were prepared in PBS; 7-B6-1-BAM diluted 1:200 and GB11-biotin diluted 1:500. 50µl per well was added and plates incubated in 37oC incubator with 5% CO2 for 1 hour. Detection antibodies were removed by flicking and wells were washed x5 with 150µl/well sterile PBS. Fluorophore conjugates were

prepared; anti-BAM-490 and SA-550 diluted 1:200 in PBS. 50μl per well was added and plates incubated in the dark at room temperature for 1 hour. Fluorophore conjugates were removed by flicking and wells were washed x5 with 150μl/well sterile PBS. 50μl per well of fluorescence enhancer (product supplied with FluoroSpot kit) was added and plates were incubated in the dark at room temperature for 15 minutes. Wells were emptied by flicking and tapping against clean paper towels before underdrains were carefully removed and plates dried under running laminar flow hood for at least 1 hour. Plates were scanned and spots counted within 48 hours using an automated ImmunoSpot plate reader (ImmunoSpot S6 Ultra, CTL).

2.9.1 Ex vivo IFNγ/Granzyme B FluoroSpot

To perform *ex vivo* FluoroSpot assays, fresh whole blood was obtained, and PBMC isolated as previously described. PBMC were resuspended in CTL test plus media at either $2x10^6$ or $2.5x10^6$ per ml, depending on available PBMC number. 100μl (200,000/250,000 cells) was added to each FluoroSpot well, on top of 50μl of CTL test plus media used to block wells, such that the total volume in each well was 150μl. Cells were then stimulated with antigen at required concentration. Positive *ex vivo* responses were defined as having at least 10 spot-forming cells (SFC) per $2x10^5$ PBMC after background subtraction, and at least double the background.

2.9.2 Cultured IFNγ/Granzyme B FluoroSpot

Primary T cell cultures were established as described in Section 2.8. 100μl from each well was added to a fresh 96-well plate and cells were then washed by added 150μl warmed R+ media and spinning at 1600 rpm for 3 minutes at room temperature. This was repeated x3. Cells were then resuspended in pre-warmed CTL test plus media, prepared as previously described. 100μl per well of cells were added to respective FluoroSpot wells on top of 50μl CTL test plus media used to block wells. Cells were then stimulated with antigen at the required concentration. Positive cultured responses were defined as having at least 20 SFC per 10^5 PBMC after background subtraction, and at least double the background.

2.10 *Ex vivo* IFNγ ELISpot

IFNγ ELISpot kits were purchased from MabTech and PVDF plates were purchased from Merck (Product Code 3420-2A and MAIPS4510). ELISpot assays were performed inside a Class II Laminar Flow Hood in sterile conditions. Plate membranes were wet with 30μl of 70% ethanol for 1 minute then washed x5 with 150μl/well sterile PBS (Thermo Fisher, Product Code 10010056). Capture antibody was prepared; 1-D1K diluted to 15μg/ml in PBS, and 50μl added per well before plates were sealed with parafilm and incubated in a fridge at 4oC overnight. Coating antibodies were removed from wells by flicking, and wells were washed x5 with 150μl/well sterile PBS. 50μl per well of CTL test plus media was added and incubated in 37oC incubator with 5% CO2 for 30 minutes. PBMC were added to corresponding wells on top of 50μl media used to block (200-250,000 cells per 100μl for *ex vivo* assays) and stimulated with peptide at the required concentration. Plates were incubated in 37oC incubator with 5% CO2 for 24 hours. Cells were removed by flicking and wells were washed x5 with 150μl/well sterile PBS. Detection antibody was prepared; 7-B6-1-biotin to 1μg/ml in PBS, 50μl added per well and plates were incubated in 37oC incubator with 5% CO2 for 1 hour. Detection antibody was removed by flicking and wells were washed x5 with 150μl/well sterile PBS. Streptavidin-ALP was prepared by at 1:1000 in PBS and 50μl added per well. Plates were incubated at room temperature for 1 hour before Streptavidin-ALP was removed by flicking and wells were washed x5 with 150μl/well sterile PBS. 50μl/well of BCIP/NBT substrate solution (MabTech, Product Code 3650-10) was added to each well for approximately 10 minutes, until plates developed distinct spots. The response to PHA was used to monitor this spot development. Colour development was stopped by washing plates extensively with tap water. Underdrains were subsequently carefully removed, and plates dried under running laminar flow hood for at least 1 hour. Plates were then scanned and counted using automated ImmunoSpot plate reader (ImmunoSpot S6 Ultra, CTL). Positive *ex vivo* responses were defined as having at least 10 spot-forming cells (SFC) per $2x10^5$ PBMC after background subtraction, and at least double the background.

2.11 Cultured IFNγ/IL-4/IL-10 FluoroSpot

IFNγ/IL-4/IL-10 tricolour FluoroSpot kits were purchased from ImmunoSpot (Catalogue Numbers hT3013F, hT02, hT36, and hT60). PVDF plates were provided with this kit, alongside all diluents required. Plate membranes were wet with 30μl of 70% ethanol for 1 minute then washed x5 with 150μl/well sterile PBS (Thermo Fisher, Product

Code 10010056). Capture antibodies were prepared; for 1 plate, 40μl IFNγ, 80μl IL-4, and 80μl IL-10 were added to 10ml of Diluent A. 80μl per well of capture antibody solution was added before plates were sealed with parafilm and incubated in a fridge at 4oC overnight. Capture antibodies were removed from wells by flicking, and wells were washed x5 with 150μl/well sterile PBS. 50μl per well of CTL test plus media was added and incubated in 37oC incubator with 5% CO2 for 30 minutes. PBMC were added to corresponding well on top of 50μl media used to block (50-100,000 cells per 100μl) and stimulated with peptide at the required concentration. Plates were incubated in 37oC incubator with 5% CO2 for 48 hours before cells were removed by flicking and wells were washed x3 with 150μl/well sterile PBS, and x2 with 150μl/well 0.05% Tween-PBS. Detection antibodies were prepared; for 1 plate, 20μl anti-IFNγ, 30μl anti-IL-4, and 30μl anti-IL-10 were added to 10ml of Diluent B and filtered through a 0.22μm filter. 80μl per well of detection antibody solution was added and plates were incubated at room temperature for 2 hours. Detection antibodies were removed by flicking and wells were washed x5 with 150μl/well 0.05% Tween-PBS. Tertiary solution (fluorophore conjugates) was prepared; for 1 plate, 25μl anti-FITC Alexa Fluor 488 (IFNγ), 25μl anti-Hapten1 CTL-Yellow (IL-10), and 50μl Step CTL-Red (IL-4) were added to 10ml Diluent C. 80μl per well of tertiary solution was added and plates incubated at room temperature for 1 hour. Tertiary solution was removed by flicking and wells were washed x5 with 150μl/well distilled water. Underdrains were then carefully removed, and the backs of the plates were washed with distilled water. Plates were dried under running laminar flow hood for at least 1 hour and scanned/counted within 48 hours using automated ImmunoSpot plate reader (ImmunoSpot S6 Ultra, CTL). Positive cultured responses were defined as having at least 20 SFC per 10^5 PBMC after background subtraction, and at least double the background.

Primary T cell cultures subsequently investigated by IFNγ/IL-4/IL-10 tricolour FluoroSpot were established from frozen TaCTiCC patient samples. In these experiments, live cell number post 14-day culture was enumerated by adding 30μl of 200μl primary T cell cultures to a fresh 96-well plate and adding 0.5μl PI as a live/dead stain. Total live cell number was ascertained as described in Section 2.6.2. This was used to normalize results seen in FluoroSpot assays to total live cell number.

2.12 Immunohistochemistry

DNAJB7 expression by Immunohistochemistry (IHC) was initially optimised manually, then was performed at high throughput on the Leica Bond RX Automated IHC Research Stainer. DNAJB7 antibody was purchased from Atlas Antibodies (Product Code HPA000534, concentration 0.05mg/ml, rabbit polyclonal IgG antibody). This was the same antibody used by the Human Protein Atlas. Isotype controls were performed using rabbit IgG polyclonal isotype control (Abcam, Product Code ab37415, concentration 5mg/ml). 5μm sections were cut from formalin-fixed paraffin embedded blocks of CRC tumour tissue and healthy colon tissue of patients previously consented to studies within the Godkin/Gallimore group. Testis and placenta tissue were used as a positive control for DNAJB7 expression; this staining is shown alongside respective isotype controls in Figure 2.6.

For optimisation, sides were dewaxed and hydrated using xylene, descending alcohol washes, and dH2O. Antigen retrieval was performed using EDTA-based pH 9.0 antigen retrieval buffer for 30 minutes. Exogenous peroxidase activity was blocked using 0.3% hydrogen peroxide (H2O2) for 30 minutes, and non-specific antibody binding was blocked using 2.5% horse serum (VectorLabs) for 30 minutes. Sections were incubated in primary antibody at a dilution of 1:75 overnight. Antibody detection was performed using ImmPRESS polymer (VectorLabs) followed by DAB (3,3'-diaminobenzidine). Slides were counterstained with Haematoxylin, dehydrated in dH2O, ascending alcohols, and xylene, mounted in DPX (distyrene, a plasticizer, and xylene), and left overnight. Slides were scanned using Slide Scanner Axio Scan.Z1 (Zeiss), and representative images taken using Zen Blue Software.

For high throughput staining using the Leica Bond, dewaxing/hydration of sections was performed using the standard machine protocol. Antigen retrieval was performed using Bond Epitope Retrieval Solution 2, the equivalent of EDTA-based pH 9.0 antigen retrieval buffer. DNAJB7 was used at a dilution of 1:100 and was incubated for 105 minutes. Antibody detection was performed using Bond Polymer Refine Detection Kit, a DAB-based polymer detection method, followed by haematoxylin counter staining. After this, sections were removed from the Leica Bond dehydrated and processed as described above.

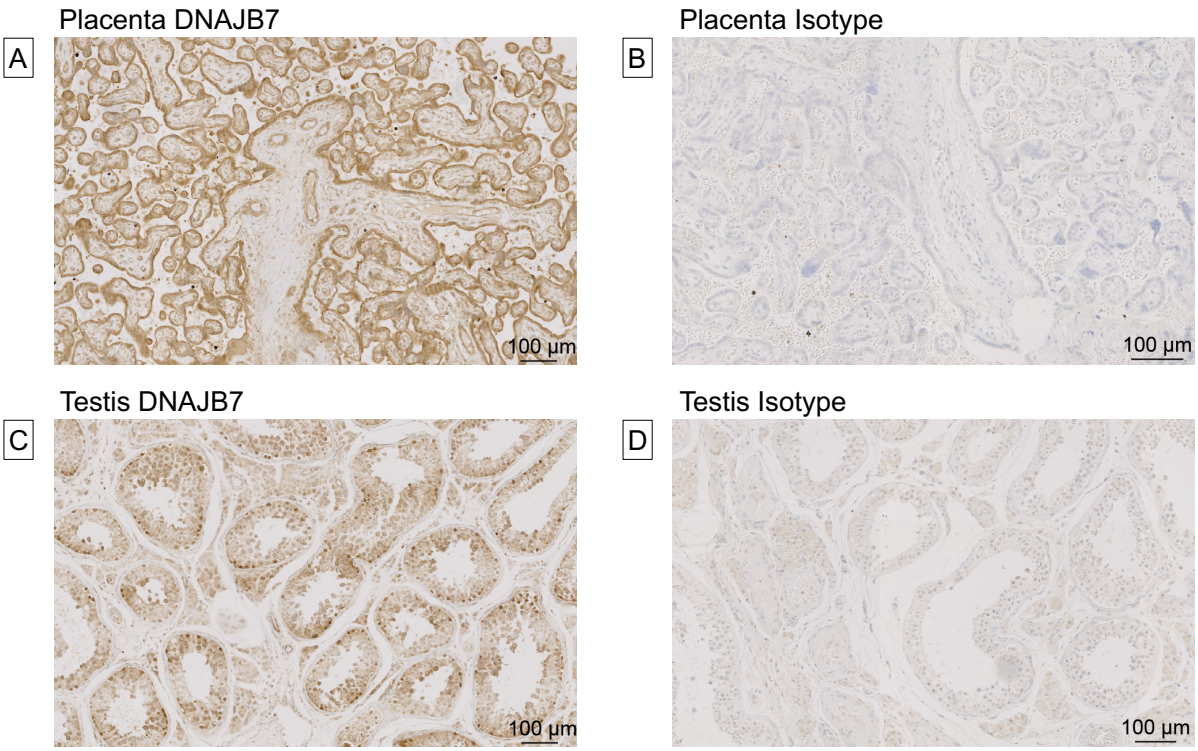


Figure 2.6 Positive control DNAJB7 staining and respective isotype controls.

DNAJB7 staining in positive control tissues placenta (A) and testis (C), alongside respective isotype controls (B) and (D) during DNAJB7 staining optimisation.

2.13 Statistical and Graphical Analysis

Statistical and graphical analysis was performed in GraphPad Prism. The normality in distribution between immune responses measured in healthy donors/CRC patients/other patients was tested by Shapiro-Wilk. Where data were normally distributed, differences between groups were compared by Welch's t-test. Where data were not normally distributed, differences were compared by Mann-Whitney U-test. Indications of significance as per Table 2.6.

Chapter 3 Identification of Plasma Protein Biomarkers to Identify Patients with Earlier-Stage and Advanced Metastatic CRC

3.1 Introduction

CRC diagnosis is complex and requires multiple approaches. Moreover, current diagnostic methods are not only invasive and time-consuming, but can prove inconclusive. There have been a number of studies into protein biomarkers for the detection and prognosis of cancer, however relatively few have progressed into clinical settings, often a result of insufficient sensitivity and/or specificity. Proteins such as CEA, cancer antigen 19-9 (CA 19-9), and C-reactive protein (CRP) are able to provide useful information on the presence of CRC, treatment response, and relapse. Their associations with other cancer types and non-cancerous conditions however, mean they are not recommended for population screening. For other cancer-types, such as prostate, biomarkers also exist with similar pitfalls, such as prostate-specific antigen (PSA). There is, therefore, a clear unmet need for reliable protein biomarkers to aid cancer diagnosis. It was hypothesised that plasma proteins may be altered between patients with earlier-stage CRC (stage I-II), those with advanced mCRC (stage IV), and healthy controls, and that these may be able to identify disease.

To address this hypothesis, the circulating levels of 31 matrix/immune proteins were measured in the plasma of 52 mCRC patients (recruited to the TaCTiCC clinical trial), 14 earlier-stage CRC patients, and 39 individuals without cancer, using Luminex Multiplex technology. Selection of proteins was based on previously reported associations with cancer and/or tumour immunity (Appendix Supplementary Table 2). The work described in this Chapter compares the plasma levels of these proteins between groups and assesses their potential as biomarkers for identifying mCRC and/or earlier-stage CRC patients. Furthermore, it explores the potential for a combination of these biomarkers to increase overall diagnostic capacity.

3.2 Results

3.2.1 Comparison of Individual Plasma Proteins Between i) Patients with Earlier-stage CRC (Stages I-III) ii) Patients with Advanced Metastatic CRC (Stage IV) and iii) Healthy Controls

The groups and measured analytes are detailed in Methods, and Appendix Supplementary Table 2. Data for IL-27 and MMP-12 are not shown as in all groups the measured plasma levels were low or undetectable in the majority of patients and controls. Results are summarised in Table 3.1.

The main difference in the levels of proteins was found between patients with mCRC and healthy controls. Higher levels of matrix proteins including MMP-3, MMP-9, MMP-10, Tenascin-C, and TIMP-1 were found in patients with mCRC compared to controls (Figure 3.1.8-3.1.10, 3.1.14, 3.1.16). Conversely, the levels of MMP-1 and MMP-13 were significantly reduced in these patients (Figure 3.1.7 and 3.1.11). Differences were also found in immune-related plasma proteins between these groups. There was a significant reduction in plasma APOA1, BDNF, IL-2, IL-15, and RANTES in patients with mCRC versus individuals without cancer (Figure 3.1.1-1.4 and 3.1.13). Furthermore, levels of IL-21, IP-10, PIGF-1, and TIM-3 were significantly increased in mCRC (Figure 3.1.5-1.6, 3.1.12, 3.1.15). These differences reached statistical significance and demonstrate widespread changes of soluble matrix and immune proteins in the context of advanced mCRC compared to healthy controls.

When metastatic and non-metastatic CRC patients were considered together and levels of plasma proteins compared to healthy controls, many of the proteins detailed above remained significantly altered, following the same pattern (data not shown). The results suggest there are changes in the plasma protein composition associated with the presence of CRC.

The levels of these proteins were compared between patients with earlier-stage CRC and healthy controls. MMP-13 and PIGF-1 were reduced in earlier-stage CRC patients, with a strong trend towards statistical significance (Figure 3.1.11-3.1.12). Furthermore, RANTES was significantly reduced, and TIMP-1 significantly increased in these patients compared to healthy controls (Figure 3.1.13 and 3.1.16).

Levels of these proteins in CRC patients with and without metastasis were next compared. APOA1, BDNF, and IL-2 were significantly lower in patients with metastatic

disease compared to those without (Figures 3.1.1-3.1.3). Additionally, the levels of MMP-3, MMP-9, Tenascin-C, and TIMP-1, were all significantly increased in patients with metastasis (Figures 3.1.8-3.1.9, 3.1.14, 3.1.16). In non-metastatic patients, these proteins were present at similar levels to healthy controls, with the exception of TIMP-1 which was significantly higher than control levels, but significantly lower than levels seen in mCRC patients (Figure 3.1.16).

There are significant differences in the levels of multiple matrix/immune-proteins and growth factors in the plasma of cancer patients compared to controls. This is particularly evident when comparing advanced mCRC patients to healthy controls. Additionally, there are differences in the plasma composition of earlier-stage CRC patients compared to healthy controls, and between CRC patients with and without metastatic disease. The levels of these 31 proteins in each group are summarised in Table 3.1. These results support that there are global serological changes associated with cancer development and progression, which are easy to measure, and may provide useful insight into disease presence and/or metastatic spread.

Plasma Protein pg/ml (Mean, 95% CI, Median)	Healthy	Earlier-Stage CRC (Stage I-III)	Metastatic CRC (Stage IV)	Change mCRC versus healthy
APOA1	1.92×10^{10} (1.67×10^{10}-2.18×10^{10}) Median 1.82×10^{10}	1.96×10^{10} (1.28×10^{10}-2.63×10^{10}) Median 1.79×10^{10}	1.23×10^{10} (9.20×10^{9}-1.53×10^{10}) Median 1.01×10^{10}	↓
BDNF	451.2 (267-635.3) Median 188.2	263.4 (47.2-479.6) Median 118.1	46.8 (26.9-66.7) Median 15.7	↓
IL-15	121.8 (45.2-198.4) Median 21.1	63.9 (-60.8-188.6) Median 0	10.6 (-4.1-25.4) Median 0	↓
IL-2	36.5 (26.9-46.1) Median 21.1	36.1 (-2.9-75.1) Median 21.1	15.6 (4.9-26.2) Median 2.1	↓
MMP-1	226.6 (120.6-332.6) Median 89.5	128.6 (66.1-191.2) Median 89	122.5 (70.7-174.3) Median 62.7	↓
MMP-13	140.9 (52.7-229) Median 91.7	51.1 (-21.5-123.7) Median 0	40.9 (-3.2-84.9) Median 0	↓
RANTES	248.6 (175.9-321.2) Median 224.4	101.8 (52.9-150.6) Median 82.7	150.4 (99.9-200.9) Median 102.2	↓

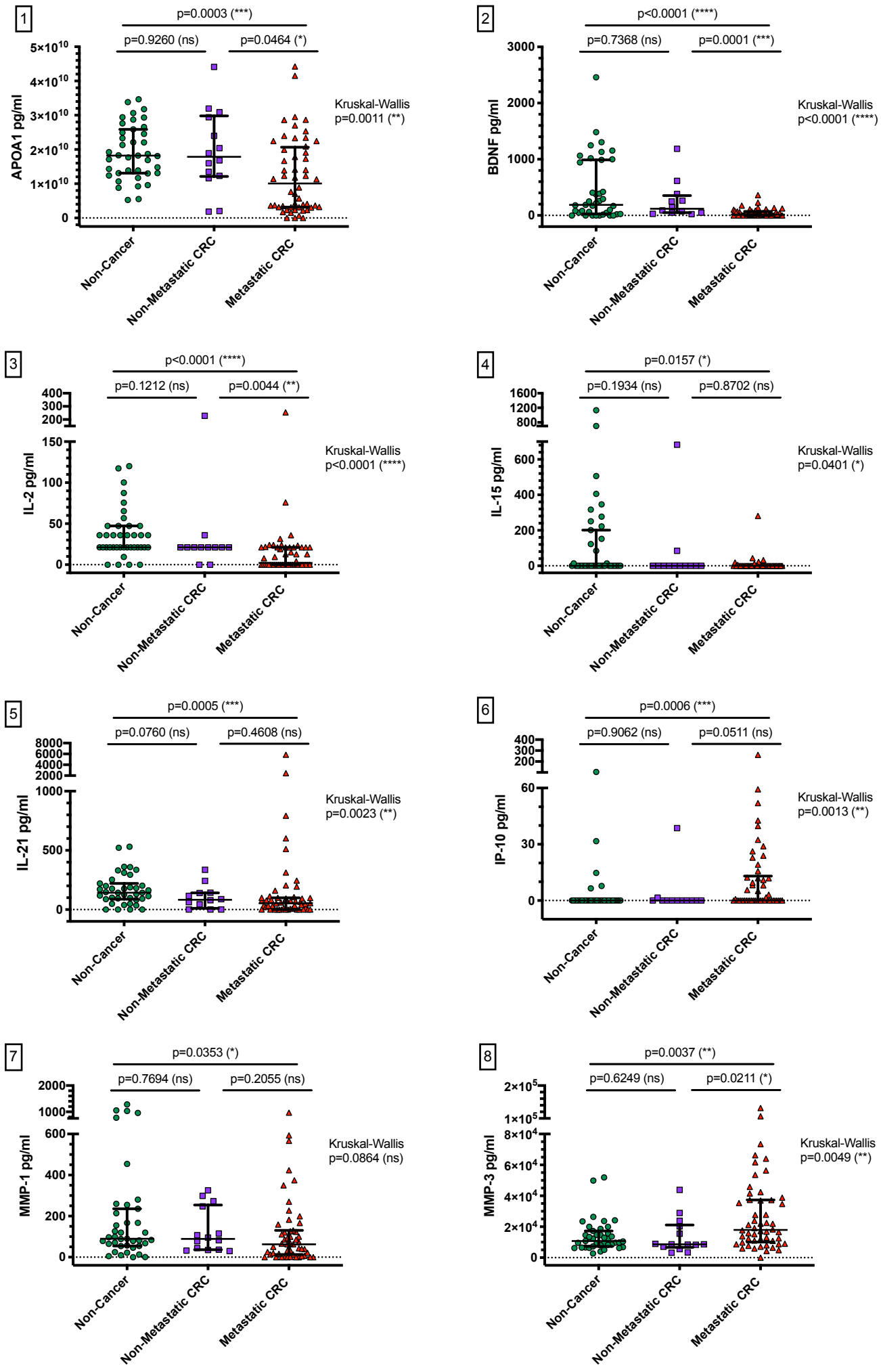
IL-21	169.8 (127.2-212.3) Median 142.3	104.2 (39.5-168.9) Median 83.4	255.2 (7.9-502.4) Median 53.9	↑
IP-10	4.2 (-1.4-9.9) Median 0	3.3 (-3.7-10.4) Median 0	14.3 (3.3-25.2) Median 0	↑
MMP-3	14129 (10721-17536) Median 10810	13909 (7217-20601) Median 8621	27667 (20439-34895) Median 17939	↑
MMP-9	2070 (1489-2651) Median 1330	2600 (1231-3969) Median 1259	3598 (2250-4945) Median 1784	↑
MMP-10	649.5 (527.5-771.6) Median 582.1	733.3 (467.9-998.7) Median 499.3	891.6 (748.2-1035) Median 783.3	↑
PIGF-1	58.4 (51.4-65.4) Median 56.6	44.1 (33.9-54.3) Median 45.6	64.8 (34.5-95.1) Median 43.9	↑
Tenascin-C	8705 (7769-9640) Median 7954	9701 (7891-11511) Median 9351	14021 (12924-15118) Median 15185	↑
TIM-3	2507 (1762-3253) Median 2187	3092 (1701-4482) Median 3547	5243 (3591-6895) Median 3378	↑
TIMP-1	1.48×10^5 (1.25×10^5-1.73×10^5) Median 1.37×10^5	1.77×10^5 (1.51×10^5-2.04×10^5) Median 1.76×10^5	3.11×10^5 (2.56×10^5-3.66×10^5) Median 2.38×10^5	↑

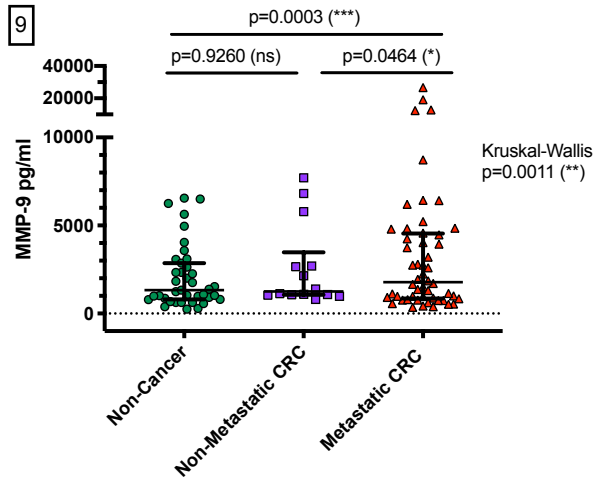
BTLA	598.9 (145.7-1052) Median 0	286 (-69.54-641.5) Median 0	459.3 (185.5-733.2) Median 0	–
CCL17	189.8 (148.2-231.3) Median 140.9	189 (105.2-272.8) Median 133.8	149.5 (113.4-185.6) Median 131.3	–
CD27	1605 (1181-2029) Median 1269	1328 (768.6-1888) Median 1059	2022 (554.4-3490) Median 928.2	–
Eotaxin	24.5 (16.1-32.9) Median 17.2	13.5 (2.1-24.8) Median 5.2	24.2 (16-32.3) Median 15.7	–
HGF	192.8 (135.7-249.9) Median 150.9	219.3 (123.1-315.6) Median 179.8	820.7 (204.7-1437) Median 207.4	–
IDO	15.8 (2.5-29) Median 0	11.2 (-3.5-25.9) Median 0	17.8 (8.4-27.2) Median 0	–
IL-22	32.9 (6.2-59.5) Median 0	29.7 (-14.7-74.1) Median 0	93.5 (-8.5-195.4) Median 0	–
MMP2	11258 (7477-15039) Median 9889	12733 (5545-19922) Median 7892	19178 (10524-27831) Median 16849	–
MMP-7	947.8 (704.2-1191) Median 775.2	753.6 (478.2-1029) Median 697.7	1535 (896.7-2173) Median 1094	–

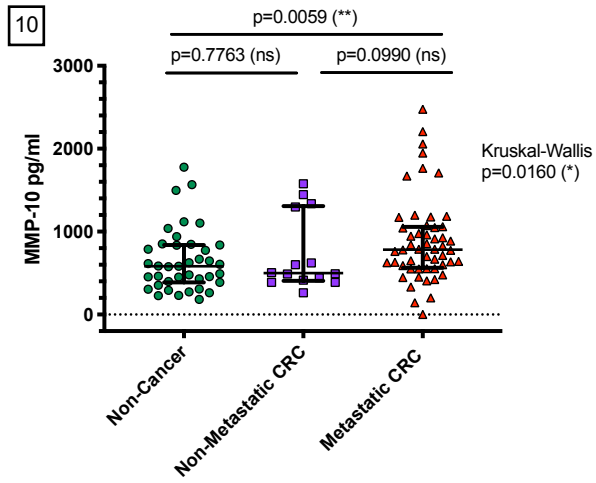
MMP-8	887.1 (560.5-1214) Median 521.8	1462 (582-2342) Median 1041	1527 (819.1-2234) Median 804.3	–
SDF1- α	682 (481.8-882.3) Median 634.6	372.7 (93.5-651.9) Median 139.2	1129 (101.5-2157) Median 389.5	–
VEGF-A	91.9 (56.9-126.8) Median 73.6	54.3 (24.9-83.6) Median 36.3	524.8 (105.4-944.2) Median 50	–
4-1BBL	411.6 (11.9-811.4) Median 0	400.9 (-465.2-1267) Median 0	565.6 (-190.3-1322) Median 0	–

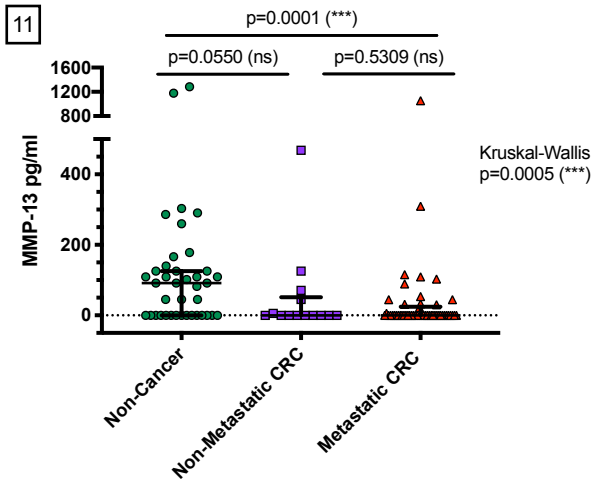
Table 3.1 Summarised plasma levels of measured proteins in individuals with non-metastatic CRC (Stages I-III), late-stage mCRC (Stage IV), and healthy controls.

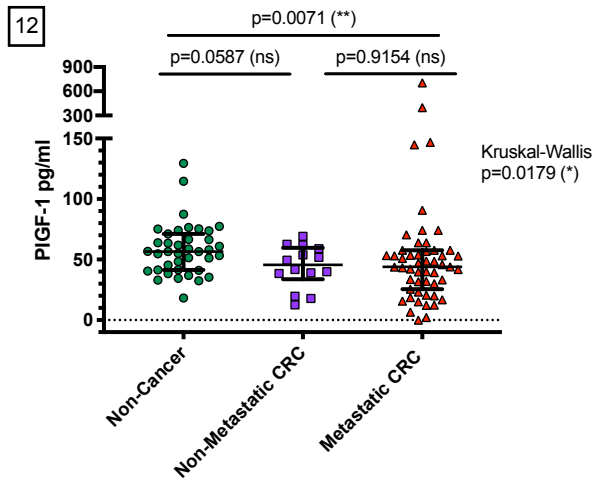
Mean, 95% confidence interval of the mean, and median pg/ml shown for each group. Directional change from levels in healthy controls to mCRC patients shown where differences were statistically significant.

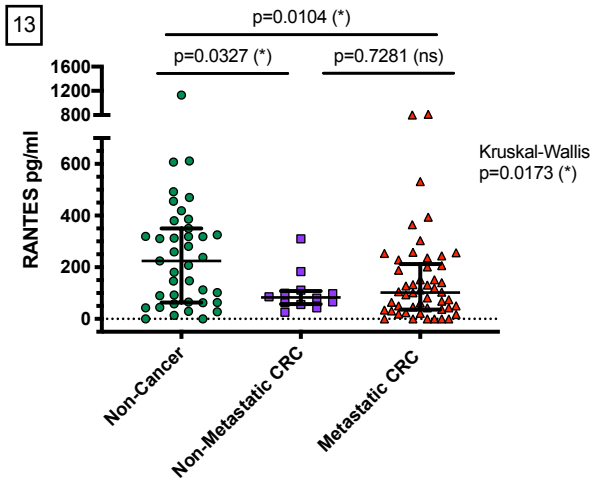

1
p=0.0003 (***)
p=0.9260 (ns)
p=0.0464 (*)
APOA1 pg/ml
5×10^10
4×10^10
3×10^10
2×10^10
1×10^10
0
Kruskal-Wallis
p=0.0011 (**)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
2
p<0.0001 (****)
p=0.7368 (ns)
p=0.0001 (***)
BDNF pg/ml
3000
2000
1000
0
Kruskal-Wallis
p<0.0001 (****)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
3
p<0.0001 (****)
p=0.1212 (ns)
p=0.0044 (**)
IL-2 pg/ml
400
300
200
150
100
50
0
Kruskal-Wallis
p<0.0001 (****)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
4
p=0.0157 (*)
p=0.1934 (ns)
p=0.8702 (ns)
IL-15 pg/ml
1600
1200
800
600
400
200
0
Kruskal-Wallis
p=0.0401 (*)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
5
p=0.0005 (***)
p=0.0760 (ns)
p=0.4608 (ns)
IL-21 pg/ml
8000
6000
4000
2000
1000
500
0
Kruskal-Wallis
p=0.0023 (**)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
6
p=0.0006 (***)
p=0.9062 (ns)
p=0.0511 (ns)
IP-10 pg/ml
400
300
200
60
30
0
Kruskal-Wallis
p=0.0013 (**)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
7
p=0.0353 (*)
p=0.7694 (ns)
p=0.2055 (ns)
MMP-1 pg/ml
2000
1000
600
400
200
0
Kruskal-Wallis
p=0.0864 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC
8
p=0.0037 (**)
p=0.6249 (ns)
p=0.0211 (*)
MMP-3 pg/ml
2×10^5
1×10^5
8×10^4
6×10^4
4×10^4
2×10^4
0
Kruskal-Wallis
p=0.0049 (**)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

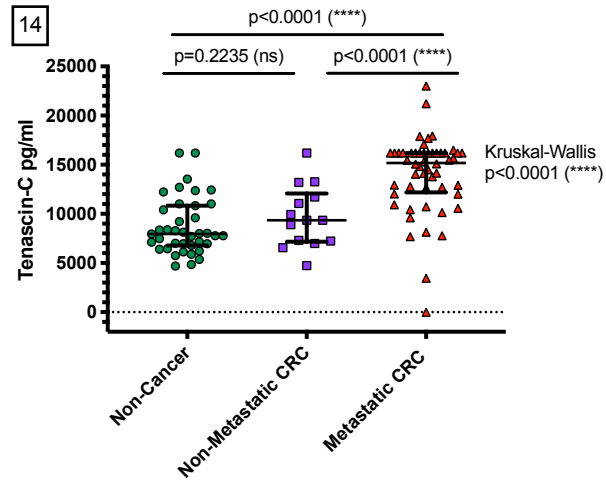

9
p=0.0003 (***)
p=0.9260 (ns)
p=0.0464 (*)
MMP-9 pg/ml
40000
20000
10000
5000
0
Kruskal-Wallis
p=0.0011 (**)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

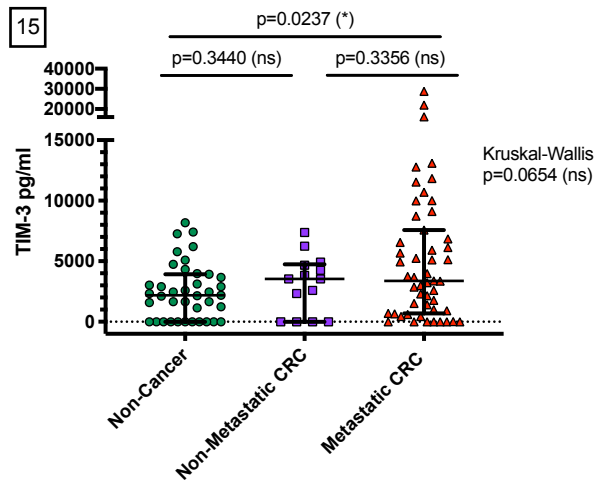

10
p=0.0059 (**)
p=0.7763 (ns)
p=0.0990 (ns)
MMP-10 pg/ml
3000
2000
1000
0
Kruskal-Wallis
p=0.0160 (*)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

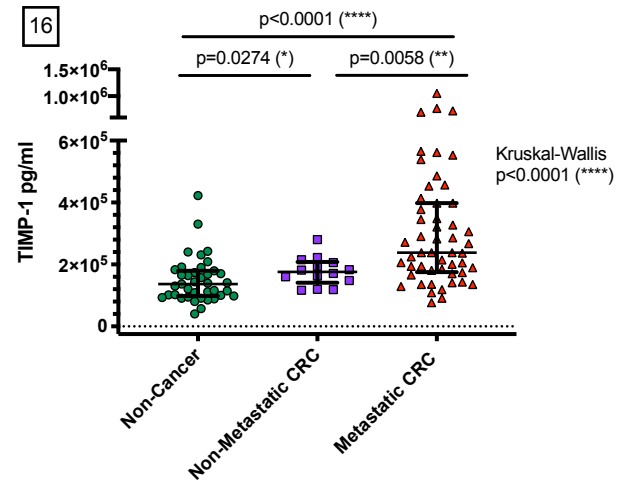

11
p=0.0001 (***)
p=0.0550 (ns)
p=0.5309 (ns)
MMP-13 pg/ml
1600
1200
800
400
200
0
Kruskal-Wallis
p=0.0005 (***)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

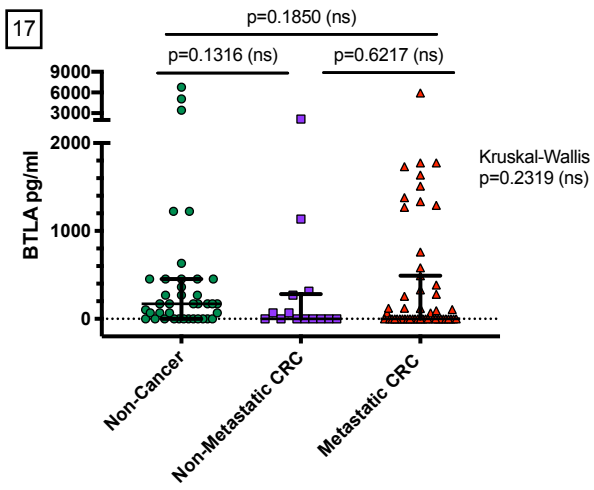

12
p=0.0071 (**)
p=0.0587 (ns)
p=0.9154 (ns)
PIGF-1 pg/ml
900
600
300
150
100
50
0
Kruskal-Wallis
p=0.0179 (*)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

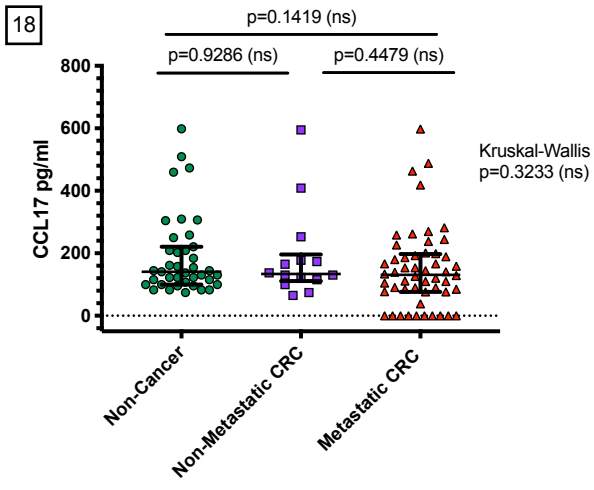

13
p=0.0104 (*)
p=0.0327 (*)
p=0.7281 (ns)
RANTES pg/ml
1600
1200
800
600
400
200
0
Kruskal-Wallis
p=0.0173 (*)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

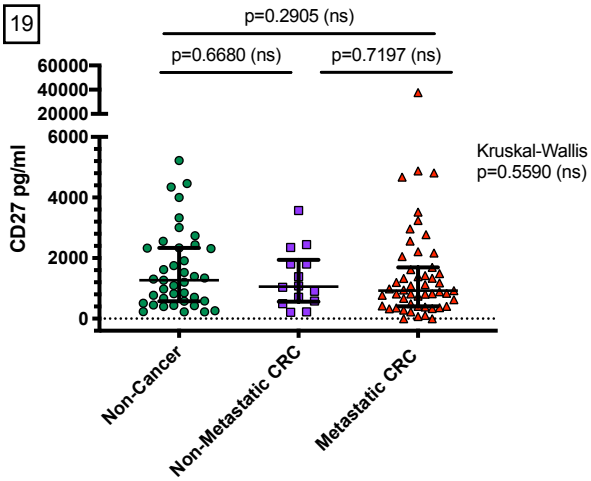

14
p<0.0001 (****)
p=0.2235 (ns)
p<0.0001 (****)
Tenascin-C pg/ml
25000
20000
15000
10000
5000
0
Kruskal-Wallis
p<0.0001 (****)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

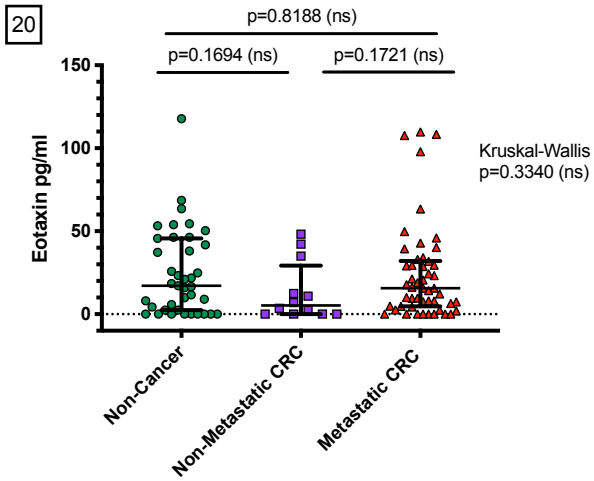

15
p=0.0237 (*)
p=0.3440 (ns)
p=0.3356 (ns)
TIM-3 pg/ml
40000
30000
20000
15000
10000
5000
0
Kruskal-Wallis
p=0.0654 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

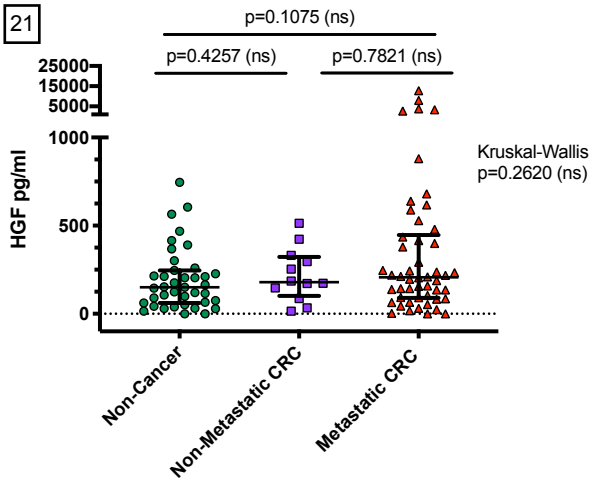

16
p<0.0001 (****)
p=0.0274 (*)
p=0.0058 (**)
TIMP-1 pg/ml
1.5×10^6
1.0×10^6
6×10^5
4×10^5
2×10^5
0
Kruskal-Wallis
p<0.0001 (****)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

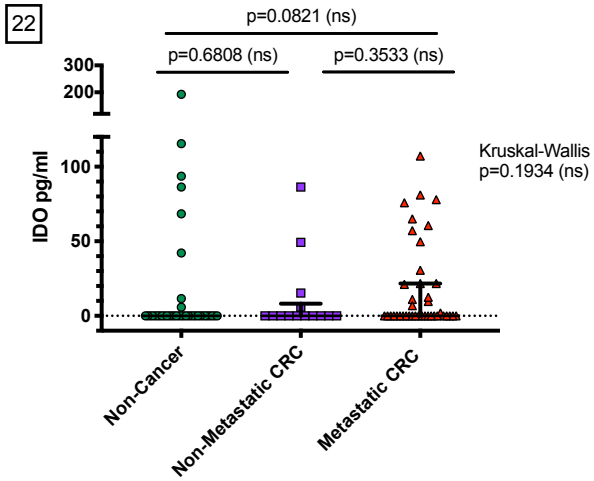

17
p=0.1850 (ns)
p=0.1316 (ns)
p=0.6217 (ns)
BTLA pg/ml
9000
6000
3000
2000
1000
0
Kruskal-Wallis
p=0.2319 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

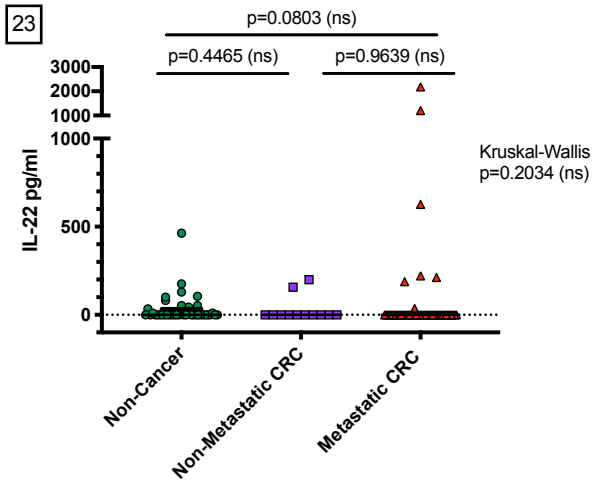

18
p=0.1419 (ns)
p=0.9286 (ns)
p=0.4479 (ns)
CCL17 pg/ml
800
600
400
200
0
Kruskal-Wallis
p=0.3233 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

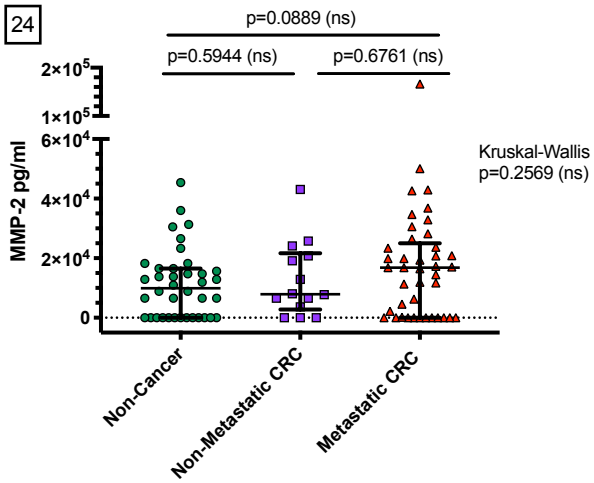

19
p=0.2905 (ns)
p=0.6680 (ns)
p=0.7197 (ns)
CD27 pg/ml
60000
40000
20000
6000
4000
2000
0
Kruskal-Wallis
p=0.5590 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC


20
p=0.8188 (ns)
p=0.1694 (ns)
p=0.1721 (ns)
Eotaxin pg/ml
150
100
50
0
Kruskal-Wallis
p=0.3340 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC


21
p=0.1075 (ns)
p=0.4257 (ns)
p=0.7821 (ns)
HGF pg/ml
25000
15000
5000
1000
500
0
Kruskal-Wallis
p=0.2620 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC


22
p=0.0821 (ns)
p=0.6808 (ns)
p=0.3533 (ns)
IDO pg/ml
300
200
100
50
0
Kruskal-Wallis
p=0.1934 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC


23
p=0.0803 (ns)
p=0.4465 (ns)
p=0.9639 (ns)
IL-22 pg/ml
3000
2000
1000
1000
500
0
Kruskal-Wallis
p=0.2034 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC


24
p=0.0889 (ns)
p=0.5944 (ns)
p=0.6761 (ns)
MMP-2 pg/ml
2×10^5
1×10^5
6×10^4
4×10^4
2×10^4
0
Kruskal-Wallis
p=0.2569 (ns)
Non-Cancer
Non-Metastatic CRC
Metastatic CRC

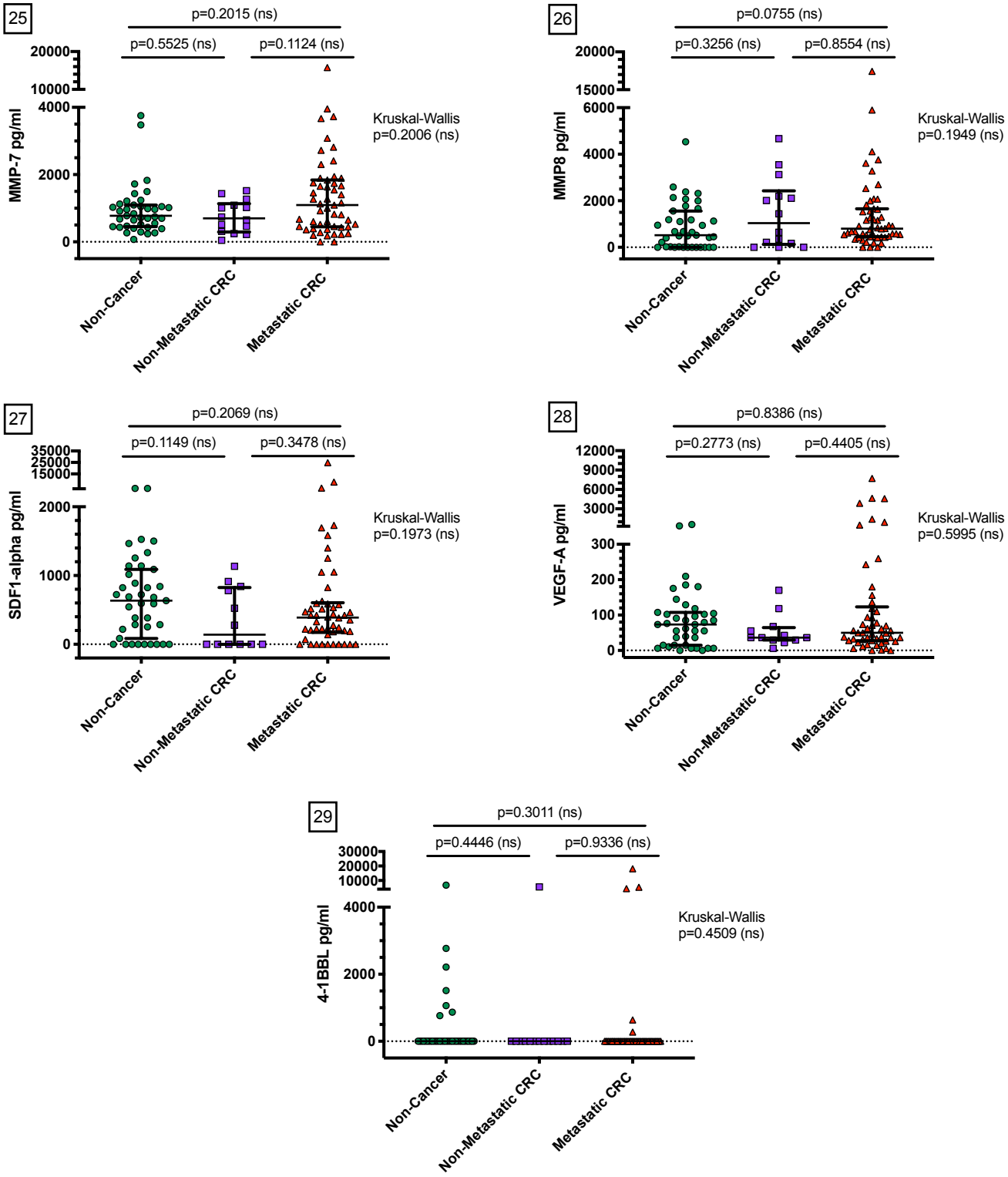


Figure 3.1 (1-29) Measured plasma proteins between patients with non-metastatic CRC (Stages I-III), advanced mCRC (Stage IV), and healthy controls.

Plasma proteins measured in frozen samples from advanced mCRC patients (n=39-52), non-metastatic CRC patients (n=12-14), and healthy controls (n=38-39). Differences between three groups were calculated using Kruskal-Wallis one-way analysis of variance, and those between two groups by Mann-Whitney U test. Group median and interquartile range shown. Asterisk indications of significance are as per methods.

3.2.2 Cluster Analysis of Plasma Proteins Between i) Patients with Earlier-stage CRC (Stages I-III) ii) Patients with Advanced Metastatic CRC (Stage IV) and iii) Healthy Controls

As shown in Section 3.2.1, there are significant differences in the levels of multiple plasma proteins between individuals with CRC and healthy controls. These are particularly evident when comparing mCRC patients with controls. Many protein biomarkers have been investigated for their ability to diagnose cancer, however, often these fail to meet the specificity and sensitivity required. It is possible that by using combinations of plasma proteins, that the ability to differentiate between mCRC patients and controls could be increased. Initially this was investigated in R by hierarchical clustering, Ward's method.

Analysis was first performed by including the 16 plasma proteins previously shown to be significantly altered between patients with mCRC and healthy controls (Table 3.1): decreased in cancer: APOA1, BDNF, IL-2, IL-15, IL-21, IP-10, PIGF-1; increased in cancer: RANTES, Tenascin-C, TIM-3, TIMP-1, MMP-1, MMP-3, MMP-9, MMP-10, and MMP-13. (IL-15 was excluded due to a high number of missing values which would have required many individuals to be excluded from the analysis). The results are shown in Figure 3.2. Three clusters emerged from these analyses. The two major clusters were able to separate mCRC patients and controls with a sensitivity of 67% (33 out of 49 cases of cancer) and a specificity of 97% (38 out of 39 healthy controls).

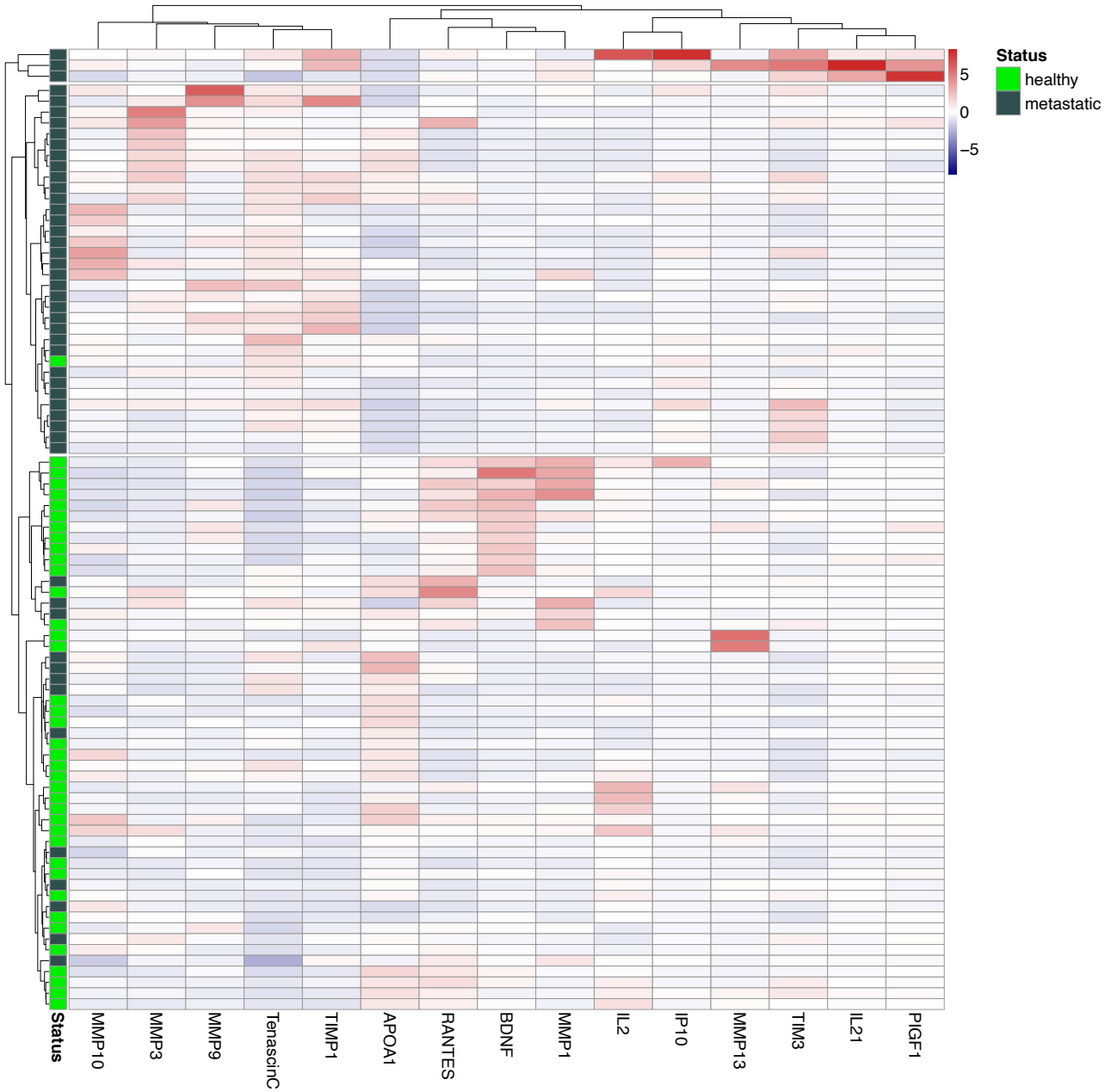


Figure 3.2 Circulating levels of plasma proteins can cluster mCRC patients and healthy controls.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of APOA1, BDNF, IL-2, IL-21, IP-10, PIGF-1, RANTES, Tenascin-C, TIM-3, TIMP-1, MMP1, MMP-3, MMP-9, MMP-10, and MMP-13 for mCRC patients (n=49) and healthy controls (n=39). These analytes were able to successfully cluster 67% (33/49) of mCRC patients into one cluster, and 97% (38/39) of healthy controls into a separate cluster. There was a third cluster of 6% (3/49) mCRC patients identified.

It was noted from Figure 3.2 that matrix proteins and immune proteins tend to cluster together, therefore it was hypothesised that by considering these two classes of proteins individually, that a more refined panel of plasma proteins best able to distinguish between mCRC and controls could also be identified.

Analysis was performed using the 7 matrix proteins which previously showed statistically significant differences between mCRC and control groups; MMP-1, MMP-3, MMP-9, MMP-10, MMP-13, Tenascin-C, and TIMP-1 (Figure 3.1). This was able to separate mCRC patients and controls with a sensitivity of 74% and a specificity of 90% (Figure 3.3). This was refined by considering combinations of 3 out of 7 of these analytes. Every possible combination of 3 out of 7 of these proteins was computed using R. Cluster analysis, Ward's method, was performed on each combination, and the number and percentage of mCRC patients and healthy control patients falling within one cluster was calculated. Using these percentages, and the calculated ratio of mCRC patients compared to controls falling within the cluster, combinations were ranked by their ability to separate the groups. Heatmaps were exported for the top 5 hits, and the best combination leading to group separation was selected. The best separation was demonstrated using MMP-13, Tenascin-C, and TIMP-1, which showed sensitivities and specificities of 86% and 79% respectively (Figure 3.4). Interestingly, when mCRC patients and the group of earlier-stage CRC patients were considered together, MMP-13, Tenascin-C, and TIMP-1 were also the three matrix proteins best able to distinguish them controls (Supplementary Figure 6).

Analysis was next performed using the 8 immune proteins previously shown to be significantly different between these groups; APOA1, BDNF, IL-2, IL-21, IP-10, RANTES, TIM-3, and PIGF-1 (Figure 3.1). Clustering failed to clearly distinguish between mCRC and controls (Supplementary Figure 7). This was also the case when considering mCRC and earlier-stage CRC patients together (Supplementary Figure 8). Multiple reduced combinations of these proteins were investigated; however, they were unable to define clear populations (data not shown).

It was hypothesised that a refined panel including matrix and immune proteins may cluster to a higher degree than the clustering shown in Figures 3.2-3.4. Using the aforementioned method, combinations of 5 out of 16 of the proteins shown to be altered between mCRC patients and controls were assessed for their ability to separate the groups. The best separation was using a combination of: IL-2, MMP-9, MMP-10, Tenascin-C, and TIM-3 (Figure 3.5). These were able to separate the two groups with a sensitivity and specificity of 92% and 82% respectively. Hence measured levels of certain

plasma proteins show a clear capacity to distinctly cluster mCRC patients and healthy controls, and it is possible to refine this panel to increase this clustering.

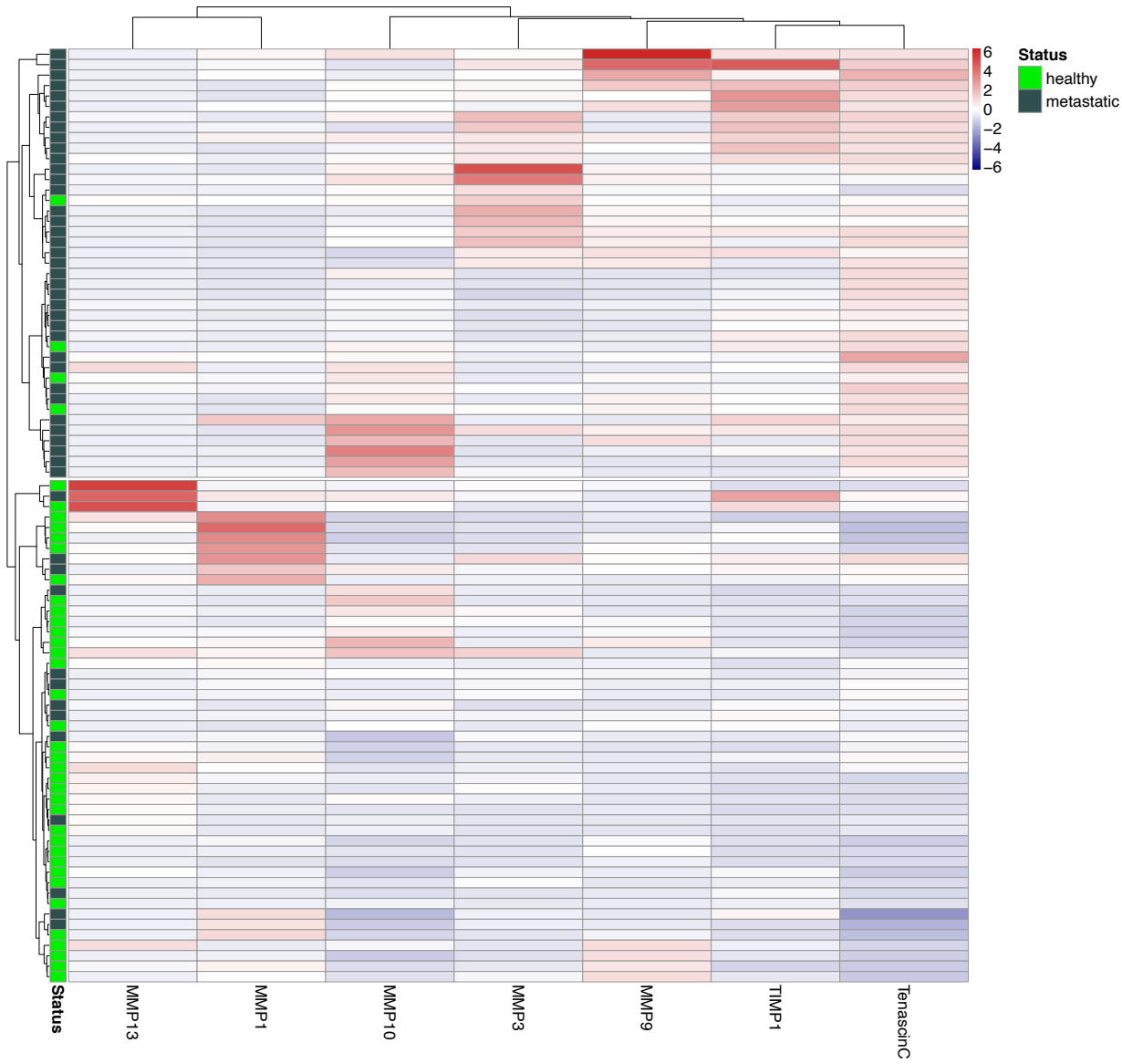


Figure 3.3 Circulating matrix proteins can cluster mCRC patients and healthy controls.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of MMP-1, MMP-3, MMP-9, MMP-10, MMP-13, Tenascin-C, and TIMP-1 for mCRC patients (n=50) and healthy controls (n=39). These analytes were able to successfully cluster 74% (37/50) of mCRC patients into one cluster, and 90% (35/39) of healthy patients into a separate cluster.

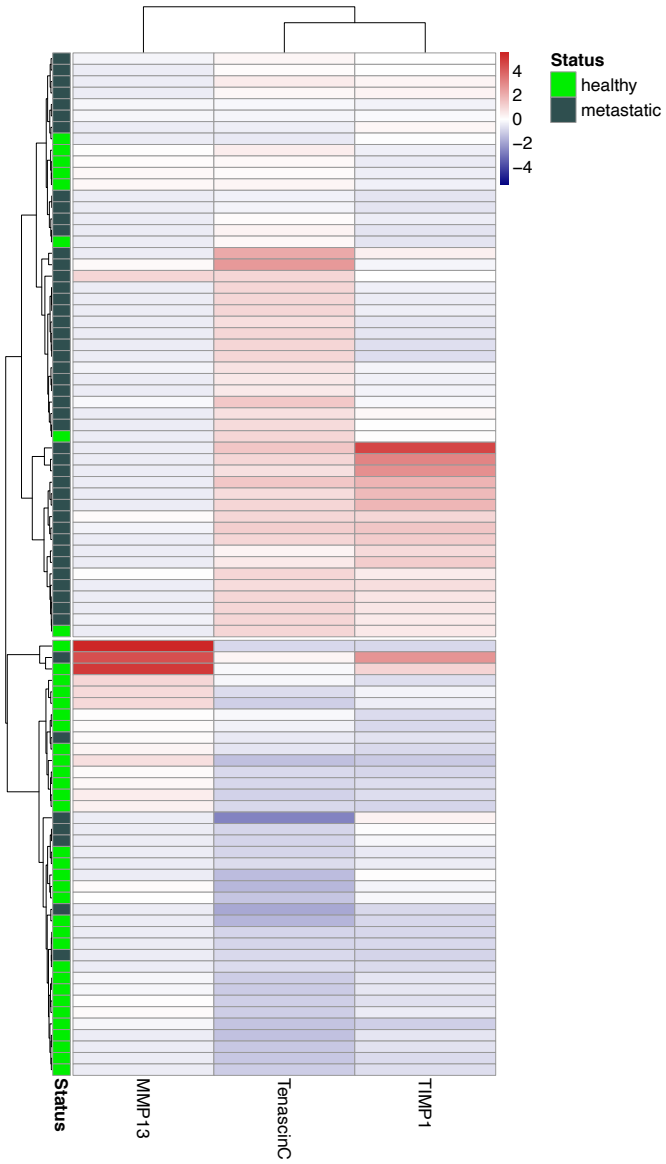


Figure 3.4 A refined panel of plasma matrix proteins MMP-13, Tenascin-C and TIMP-1 can cluster mCRC patients and healthy controls.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of MMP-13, Tenascin-C, and TIMP-1 for mCRC patients (n=50) and healthy controls (n=39). These analytes were able to successfully cluster 86% (43/50) of mCRC patients into one cluster, and 79% (31/39) of healthy controls into separate clusters.

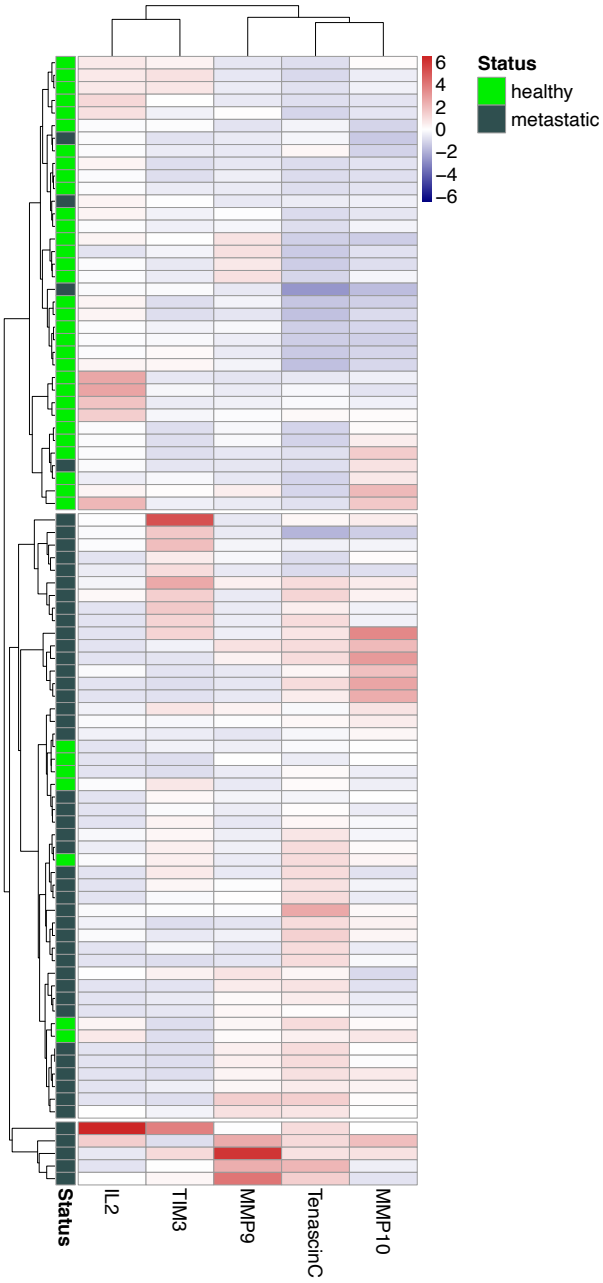


Figure 3.5 A reduced panel of five plasma proteins can cluster mCRC patients and healthy controls more effectively.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of IL-2, MMP-9, MMP-10, Tenascin-C, and TIM-3. These analytes were able to successfully cluster 82% (41/50) of mCRC patients into one cluster and 10% (5/50) mCRC patients into a second cluster. Combined this separated 92% (46/50) mCRC patients. 82% (32/39) of healthy controls were separately clustered i.e. specificity.

The ability of plasma proteins to cluster CRC patients based on the presence of metastasis is clearly shown above. A further interesting question, when reviewing the data from Figure 3.1, is whether a similar approach may be able to distinguish earlier-stage CRC patients compared to healthy controls, or even vs mCRC. Figure 3.1 shows that there are significant differences in several plasma proteins between CRC patients with and without distant metastasis. However, when including all proteins shown to be altered between these groups; APOA1, BDNF, IL-2, MMP-3, MMP-9, Tenascin-C, and TIMP-1, there was no obvious clustering pattern (data not shown). When only considering MMP-3, MMP-9, Tenascin-C, and TIMP-1, proteins associated with the extracellular matrix, clustering was able to separate mCRC patients from earlier-stage CRC patients with a sensitivity of 42% and specificity of 100% (Supplementary Figure 9). Considering immune-proteins APOA1, BDNF, and IL-2 independently showed no differentiation between patients with and without metastasis (data not shown).

As the group size of non-metastatic patients is relatively small, it is possible that important significant differences between these groups may be missed. When every protein measured by Luminex (n=31) was considered, regardless of previous differences between groups as detailed in Figure 3.1, the best combination of 3 proteins able to separate patients based on metastasis were BDNF, Tenascin-C, and TIM-3. This was able to separate with a sensitivity of 82%, and a specificity of 83% (Figure 3.6).

The circulating levels of MMP-13, PIGF-1, RANTES, and TIMP-1 are altered between healthy controls, and patients with earlier-stage CRC (Figure 3.1). For RANTES, and TIMP-1 these changes are statistically significant, and for MMP-13 and PIGF-1 they are close to significance. When considered together, these proteins were able to separately cluster 92% (11 out of 12) earlier-stage CRC patients and 64% (25 out of 39) of healthy controls (Figure 3.7).

These results indicate a significantly altered protein signature in the presence of CRC compared to non-cancer, which is further pronounced in patients with mCRC. Matrix proteins alone are able to clearly separate mCRC patients from healthy controls. Conversely, immune proteins alone are unable to clearly distinguish these groups, however, can provide useful information in combination with matrix proteins. Using multiple proteins, it is possible to identify a larger number of cancer patients from controls. Furthermore, there are significant changes in levels of some of these proteins in the plasma of CRC patients with and without metastasis, and between earlier-stage CRC patients compared to healthy controls.

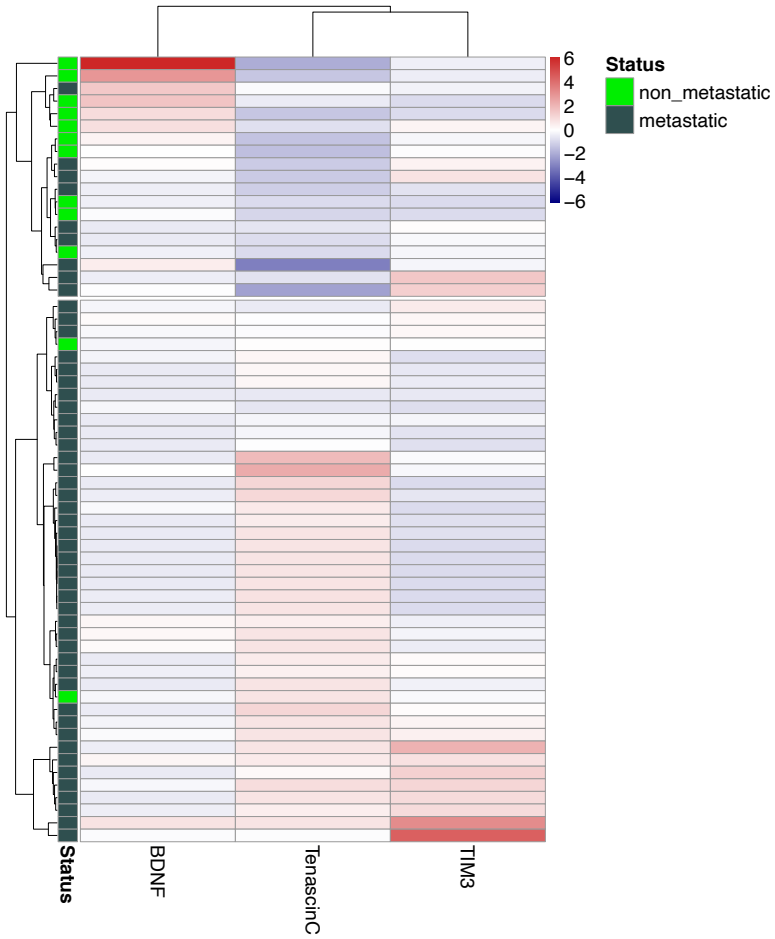


Figure 3.6 Levels of plasma BDNF, Tenascin-C, and TIM3 are able to cluster a large proportion of metastatic and non-metastatic CRC patients into distinct groups.

Hierarchical clustering was performed in R using Ward's method. The pattern of plasma levels of BDNF, Tenascin-C, and TIM3 were compared in mCRC patients (n=50) and non-metastatic CRC patients (n=12). This was able to cluster 82% (41/50) of mCRC patients, and 83% (10/12) non-metastatic patients into distinct clusters.

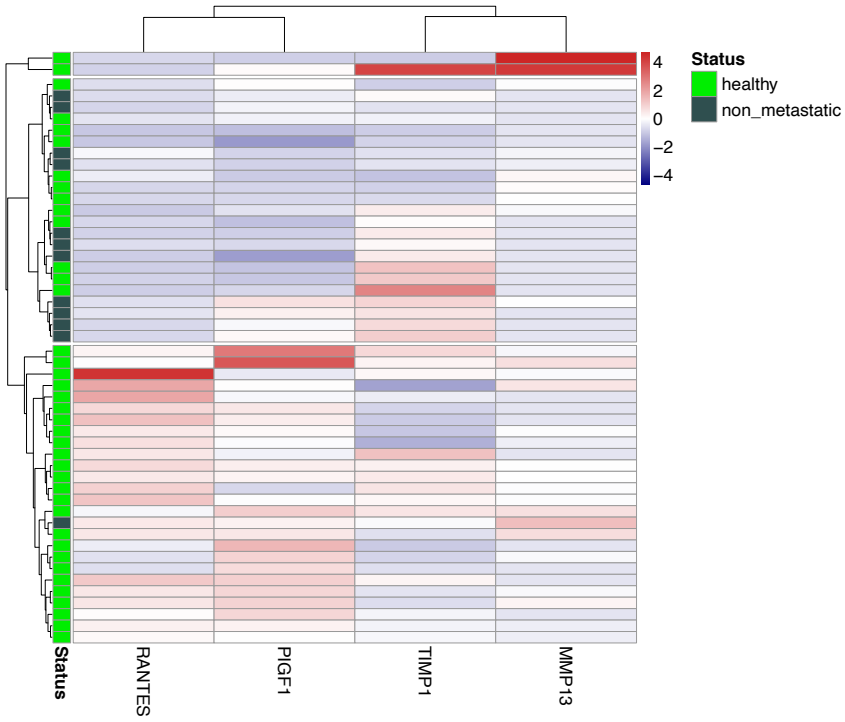


Figure 3.7 Levels of plasma MMP-13, PIGF-1, RANTES, and TIMP-1 are able to distinctly cluster a population of healthy controls from earlier-stage CRC patients.

Hierarchical clustering was performed in R using Ward's method. The pattern of plasma levels of MMP-13, PIGF-1, RANTES, and TIMP-1 were compared in earlier-stage CRC patients (stage I-III, n=12) and healthy controls (n=39). This was able to cluster 92% (11/12) of earlier-stage CRC patients, and 64% (25/39) healthy controls into distinct clusters.

3.2.3 Individual Plasma Proteins as Potential Biomarkers to Identify Advanced mCRC Patients (Stage IV)

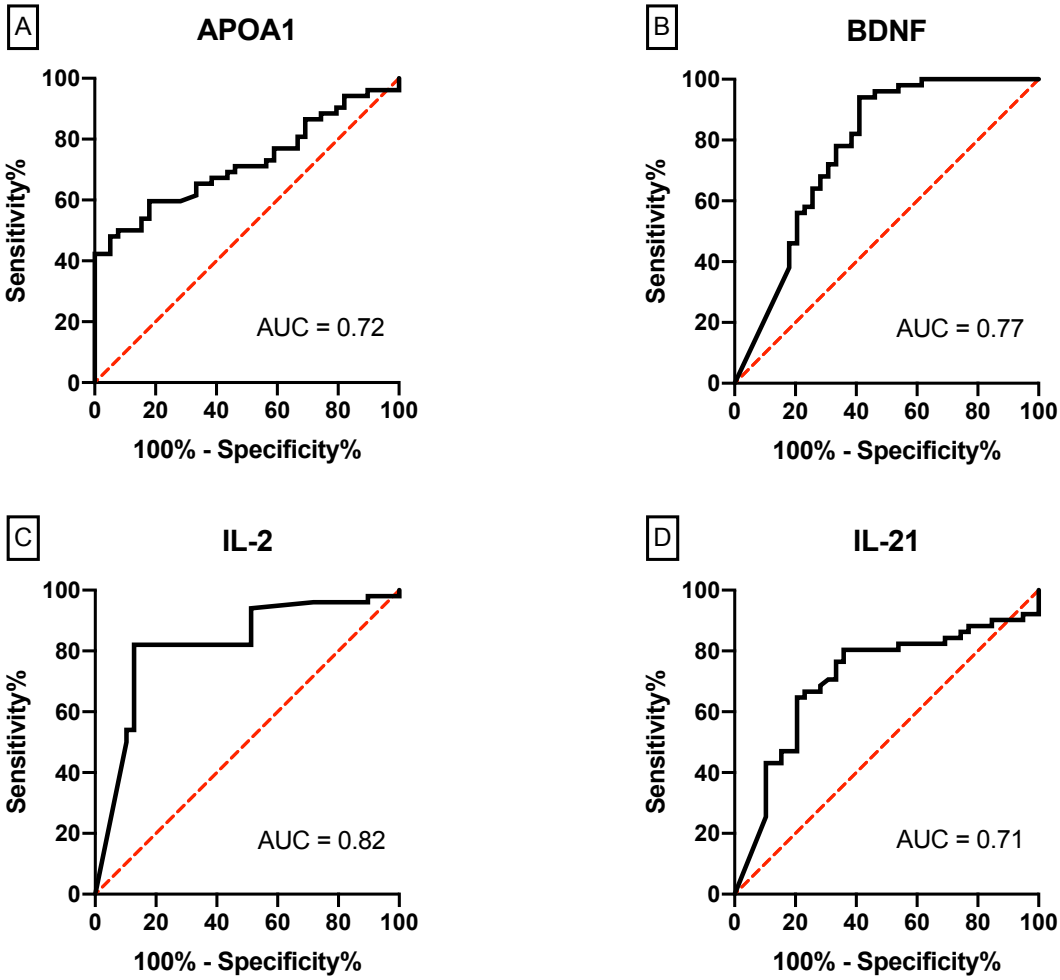
It has been shown that APOA1, BDNF, IL-2, IL-15, IL-21, IP-10, MMP-1, MMP-3, MMP-9, MMP-10, MMP-13, PIGF-1, RANTES, Tenascin-C, TIM-3, and TIMP-1, are significantly altered between patients with advanced mCRC and healthy controls (Figure 3.1). Cluster analysis has also shown that a combination of these proteins can separate these patient groups to a notably high degree of sensitivity and specificity. In order to understand how these proteins would individually perform in a diagnostic test for mCRC, receiver-operating characteristic (ROC) curve analysis was first performed. ROC analysis allows the assessment of diagnostic sensitivity and specificity of each protein at a selection of a cut-off values, and the associated area under the curve (AUC) is a measure of overall ability to distinguish between disease and healthy.

ROC analysis was performed for each of the aforementioned plasma proteins, between mCRC patients (n=50-52) and healthy controls (n=39). These potential mCRC biomarkers performed at a range of sensitivities, specificities, and cut-off values. Youden's index was therefore used to assess the overall discriminative power of each plasma protein, and to assign a cut-off value with maximum sensitivity and specificity. The relationship between the area under the ROC curve and diagnostic accuracy was assessed as described in Methods Table 2.7, ranging from "excellent" to "bad", and is summarised in Table 3.2. Although previously shown to be significantly altered between the groups, IL-15, IP-10, PIGF-1, RANTES, TIM-3, MMP-1, MMP-3, MMP-9, and MMP-10 did not show good diagnostic ability for mCRC. APOA1, BDNF, IL-21, and MMP-13, however, showed good diagnostic capacity (Table 3.2, Figure 3.8A, B, C, G). Moreover, IL-2, Tenascin-C, and TIMP-1, showed very good diagnostic ability (Table 3.2, Figure 3.8D-F). Of these proteins, the best overall individual biomarker for mCRC was Tenascin-C, with an AUC of 0.87, and sensitivity and specificity of 75% and 89.74% respectively, at a cut-off value of 12,535pg/ml.

Plasma Protein	AUC (95%CI, p-value)	Sensitivity (%)	Specificity (%)	Cut-Off Value pg/ml	Diagnostic Accuracy (AUC) mCRC
APOA1	**0.72** (0.62 to 0.82 p=0.0003)	**48.08**	**94.87**	**8.205 mg/ml**	**good**
BDNF	**0.77** (0.67 to 0.88, p<0.0001)	**94.00**	**58.97**	**154.50**	**good**
IL-2	**0.82** (0.73 to 0.92, p<0.0001)	**82.00**	**87.18**	**21.07**	**very good**
IL-15	0.63 (0.50 to 0.75, p=0.0557)	97.44	33.33	63.89	**sufficient**
IL-21	**0.71** (0.60 to 0.82, p=0.0007)	**80.39**	**64.10**	**112.60**	**good**
IP-10	0.67 (0.56 to 0.79, p=0.0049)	48.00	87.18	0.3650	**sufficient**
MMP-1	0.63 (0.52 to 0.74, p=0.0357)	45.10	82.05	44.36	**sufficient**
MMP-3	0.68 (0.57 to 0.79, p=0.0040)	59.62	74.36	15766	**sufficient**
MMP-9	0.57 (0.45 to 0.68, p=0.2825)	34.62	84.62	3660	**bad**

MMP-10	0.67 (0.56 to 0.78, p=0.0062)	71.15	61.54	625.7	**sufficient**
MMP-13	**0.71** (0.60 to 0.83, p=0.0006)	**86.00**	**61.54**	**44.64**	**good**
PIGF-1	0.67 (0.55 to 0.78, p=0.0075)	72.55	58.97	54.31	**sufficient**
RANTES	0.66 (0.54 to 0.77, p=0.0109)	88.00	46.15	258.4	**sufficient**
Tenascin-C	**0.87** (0.79 to 0.94, p<0.0001)	**75.00**	**89.74**	**12535**	**very good**
TIM-3	0.64 (0.43 to 0.74, p=0.3303)	41.18	87.18	5101	**sufficient**
TIMP-1	**0.82** (0.73 to 0.90, p<0.0001)	**69.23**	**84.62**	**193029**	**very good**

Table 3.2 ROC curve analysis for plasma proteins significantly altered between mCRC (n=39-52) and healthy controls (n=39).


A
APOA1
Sensitivity%
100% - Specificity%
AUC = 0.72
B
BDNF
AUC = 0.77
C
IL-2
AUC = 0.82
D
IL-21
AUC = 0.71
0
20
40
60
80
100

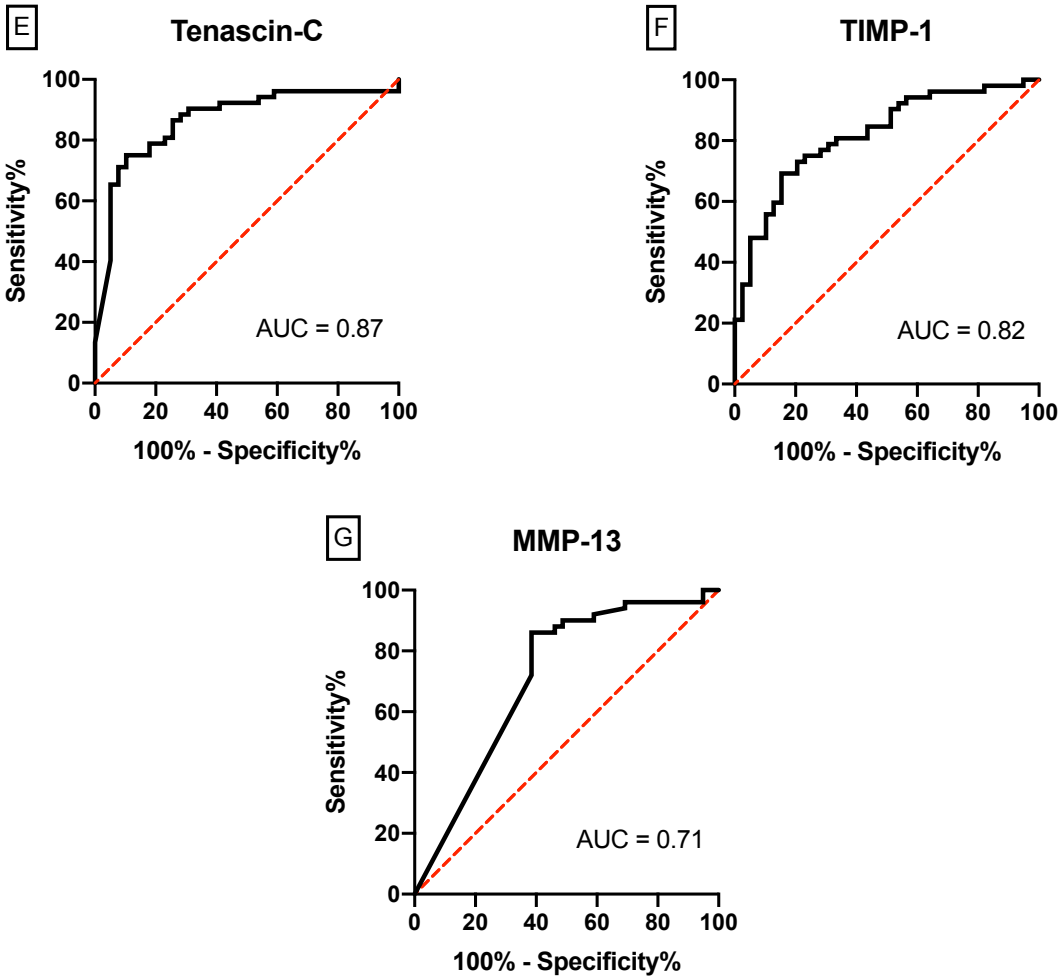


Figure 3.8 (A-G) Individual ROC curves of the plasma proteins with the best diagnostic ability for mCRC.

ROC curves were generated APOA1, BDNF, IL-2, IL-21, Tenascin-C, TIMP-1, and MMP-13 are using plasma levels measured in mCRC patients (n=50-52) and healthy controls (n=39). Area under curve as shown.

ROC curve analyses were also performed to assess the best individual biomarkers for identifying earlier-stage CRC compared to healthy controls, and for identifying metastatic CRC compared to earlier-stage CRC. MMP-13 and PIGF-1 showed sufficient diagnostic capacity for earlier-stage CRC compared to controls, both with AUC of 0.67. RANTES and TIMP-1 both showed good diagnostic capacity, with AUC of 0.71 and 0.70 respectively (Figure 3.9 A-D). At a cut-off value of 111.7 pg/ml, RANTES had a sensitivity of 83.33% and a specificity of 64.1%, whereas at a cut-off value of 1.16x105 pg/ml, TIMP-1 had a sensitivity of 100% and a specificity of 43.59%. These are summarised in Table 3.3.

When assessing individual biomarkers for the ability to diagnose between mCRC and earlier-stage disease, APOA1 showed sufficient diagnostic ability, whereas IL-2, MMP-3, and TIMP-1 had good ability, and BDNF and Tenascin-C had very good ability. These results are summarised in Table 3.4, and individual ROC curves are shown in Figure 3.10. At a cut-off value of 21.63 pg/ml, BDNF had a sensitivity of 58% and a specificity of 100%. Tenascin-C had a sensitivity and specificity of 65.38% and 92.86% respectively, at a cut-off of 13531pg/ml. BDNF and Tenascin-C both had AUC of 0.83 (Figure 3.10).

Plasma Protein	AUC (95%CI, p-value)	Sensitivity (%)	Specificity (%)	Cut-Off Value pg/ml	Diagnostic Accuracy (AUC) Earlier-Stage CRC
MMP-13	0.67 (0.50-0.83 p=0.0679)	85.71	53.85	76.52	**sufficient**
PIGF-1	0.67 (0.51-0.83, p=0.0579)	71.43	58.97	54.33	**sufficient**
RANTES	**0.71** (0.57-0.84, p=0.0330)	**83.33**	**64.1**	**111.7**	**good**
TIMP-1	**0.70** (0.56-0.84, p=0.0279)	**100**	**43.59**	**1.16 x 10^5**	**good**

Table 3.3 ROC curve analysis for plasma proteins significantly altered between earlier-stage CRC (n=12-14) and healthy controls (n=39).

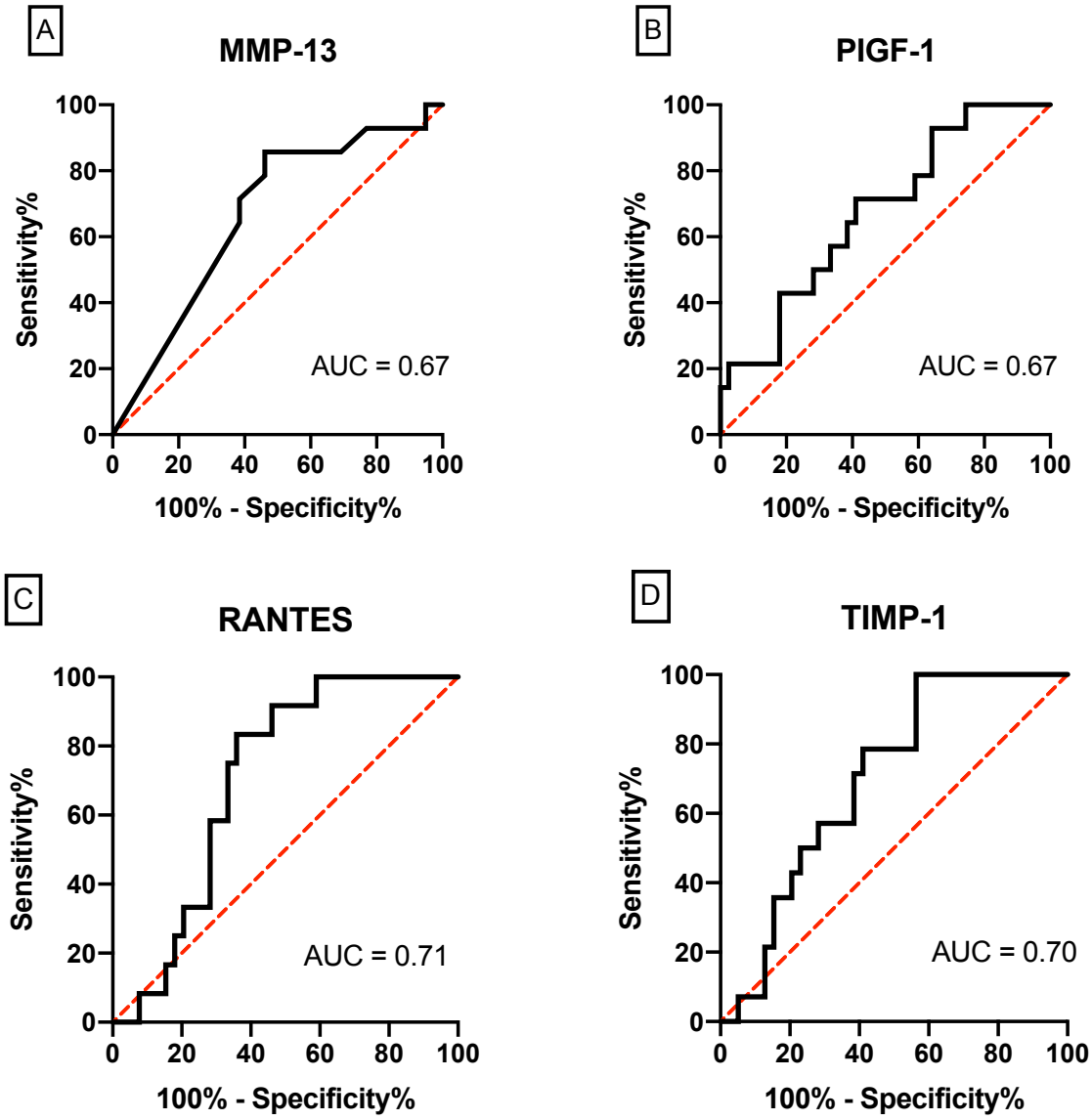


Figure 3.9 (A-D) Individual ROC curves of the plasma proteins with the diagnostic capacity for earlier-CRC.

ROC curves were generated MMP-13, PIGF-1, RANTES, and TIMP-1, using plasma levels measured in earlier-CRC patients (n=12-14) and healthy controls (n=39). Area under curve as shown.

Plasma Protein	AUC (95%CI, p-value)	Sensitivity (%)	Specificity (%)	Cut-Off Value pg/ml	Diagnostic Accuracy (AUC) mCRC
APOA1	0.67 (0.51-0.84 p=0.0464)	53.85	85.71	1.15 x10^{10}	**sufficient**
BDNF	**0.83** (0.72-0.95, p=0.0004)	**58**	**100**	**21.63**	**very good**
IL-2	**0.75** (0.59-0.91, p=0.0077)	**82**	**83.33**	**21.07**	**good**
MMP-3	**0.70** (0.55-0.85, p=0.0220)	**78.85**	**64.29**	**8966**	**good**
Tenascin-C	**0.83** (0.71-0.94, p=0.0002)	**65.38**	**92.86**	**13531**	**very good**
TIMP-1	**0.74** (0.62-0.86, p=0.0067)	**57.69**	**92.86**	**2.24 x 10^5**	**good**

Table 3.4 ROC curve analysis for plasma proteins significantly altered between earlier-stage CRC (n=12-14) and mCRC (n=50-52).

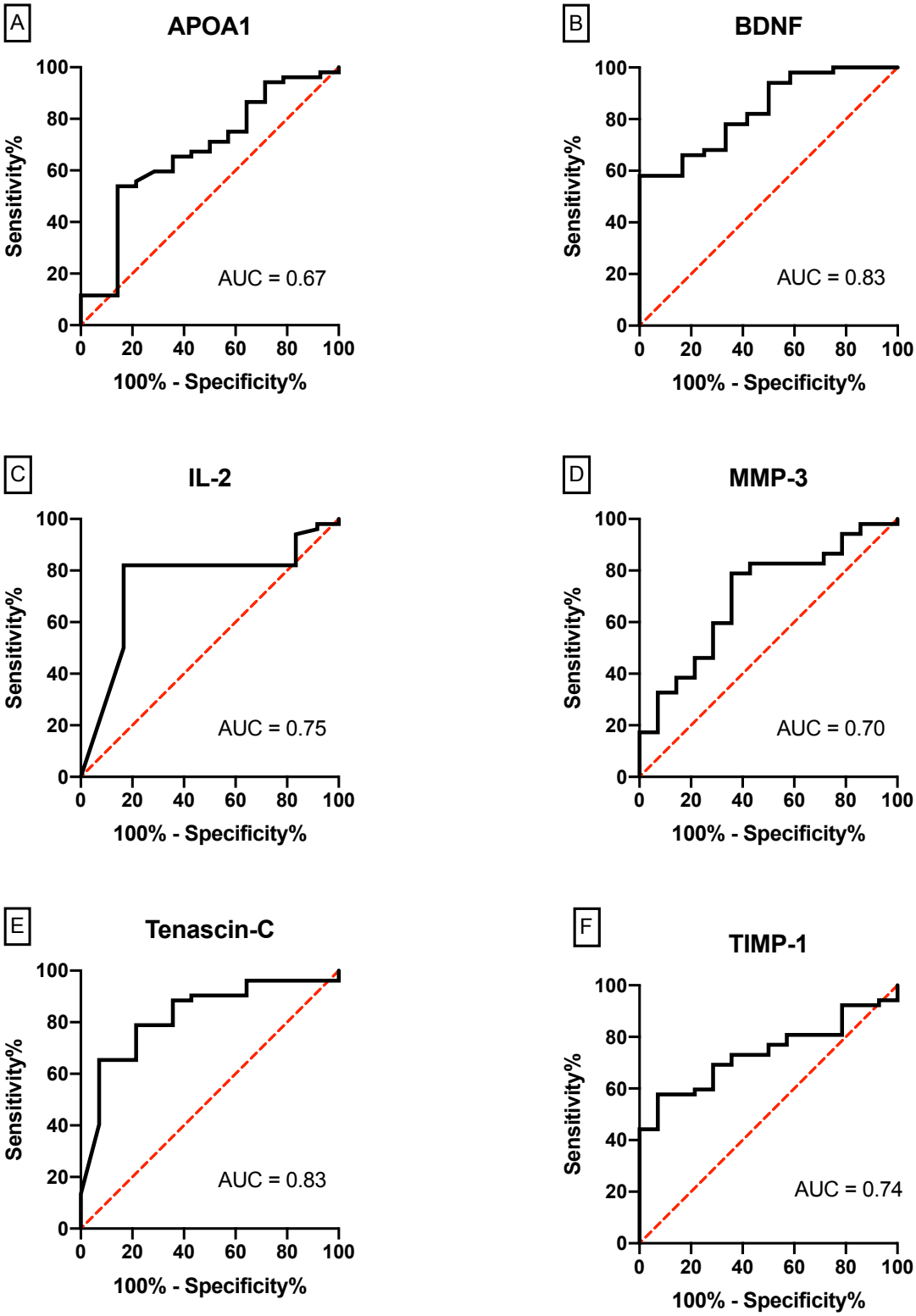


Figure 3.10 (A-F) Individual ROC curves of the plasma proteins with the capacity to diagnose mCRC patients from earlier-stage CRC patients.

ROC curves were generated APOA1, BDNF, IL-2, MMP-3, Tenascin-C, and TIMP-1 using plasma levels measured in mCRC patients (n= 50-52), and earlier-CRC patients (n=12-14). Area under curve as shown.

3.2.4 Modelling a Panel of Potential Plasma Protein Biomarkers to Identify Advanced mCRC (Stage IV) and Earlier-Stage mCRC (Stage I-III) Patients

The above two results sections identify different approaches to employing the levels of plasma proteins to identify patients with advanced metastatic CRC. Narrowing down from 31 to just 5 proteins (IL-2, MMP-9, MMP-10, Tenascin-C, and TIM-3), cluster analysis identified mCRC patients with a sensitivity of 92% and specificity of 82%. Analysing individual proteins using ROC curves and cut-off values using Youden's Index showed good sensitivity and specificity of individual proteins for identifying mCRC patients, for example Tenascin-C with a sensitivity of 75% and specificity of 89%.

To explore these data further, logistic regression analysis was performed, with the binary outcome dependent variable (healthy vs advanced cancer) to create a model for identifying mCRC patients compared to healthy controls. A logistic regression model was built by initially including the measurements of all proteins known to be significantly altered between mCRC patients and controls: APOA1, BDNF, IL-2, IL-21, IP10, MMP-1, MMP-3, MMP-9, MMP-10, MMP-13, PIGF-1, RANTES, Tenascin-C, TIM-3, TIMP-1. When including all of these proteins, many of them did not show statistical significance within the model, therefore the number of parameters was reduced using a backwards selection approach, by omitting the least significant protein at each stage, until every variable contained was significant. This resulted in a model containing APOA1, IL-2, Tenascin-C, and TIMP-1 (Table 3.5). ROC analysis was performed using the logistic predictor values calculated using this model, resulting in an AUC of 0.92, $p<0.0001$ (Figure 3.11A). Youden's Index was calculated to find the maximum sensitivity and specificity allowed by the model; these were 84% and 90% respectively. Spearman's correlation coefficient revealed a moderate positive correlation between Tenascin-C and TIMP-1, therefore models omitting either of these variables were also tested. Omission of either Tenascin-C or TIMP-1 from the model resulted in a reduced AUC, and reduced sensitivity and specificity as calculated by Youden's Index. The accuracy of the model containing variables APOA1, IL-2, Tenascin-C, and TIMP-1, was next tested by cross-validation. 75% (n=60) of total patients and controls were randomly assigned to train the logistic regression model. This resulted in an accuracy of 83% for the train dataset. The accuracy of the model was then assessed on the remaining 25% of individuals (n=29) resulting in an accuracy of 90%. It should be noted that assignment to the train and test dataset is random, therefore varied accuracies are observed each time this is performed; this is the result after 1 repetition.

As shown in Figure 3.4, the three matrix proteins best able to separate mCRC patients from controls by cluster analysis are; MMP-13, Tenascin-C, and TIMP-1. Furthermore, each of these proteins individually had good to very good ability to identify mCRC patients from controls (Table 3.2, Figure 3.8E-G). It was hypothesised that a logistic regression model including these proteins would better identify mCRC patients compared to each protein individually. Using these proteins, a logistic regression model was built (Table 3.6). ROC analysis was performed using the logistic predictor values obtained from the model, resulting in an AUC of 0.91, $p<0.0001$ (Figure 3.11B). Youden's Index identified sensitivity and specificity of 86% and 90% respectively. As previously described, Spearman's correlation coefficient revealed a moderate positive correlation between Tenascin-C and TIMP-1, therefore two further models were explored; MMP-13 and Tenascin-C, and MMP-13 and TIMP-1. These models resulted in a reduced AUC, and reduced sensitivity and specificity as calculated by Youden's Index, compared to the model containing all three variables. The model containing MMP-13, Tenascin-C, and TIMP-1 was then cross-validated as described above, resulting in an accuracy of 83% for the train dataset (n=53), and 89% for the test dataset (n=36).

Cluster analysis has shown that the 5 proteins best able to separate mCRC patients and controls were: IL-2, MMP-9, MMP-10, Tenascin-C, and TIM-3 (Figure 3.5). When including all 5 of these proteins in a logistic regression model, MMP-9 and MMP-10 did not show statistical significance within the model, therefore were omitted. The resulting model included variables IL-2, Tenascin-C, and TIM-3 (Table 3.7). The logistic predictor values obtained from the model were used to perform ROC curve analysis, resulting in an AUC of 0.92, $p<0.0001$ (Figure 3.11C). Youden's Index identified sensitivity and specificity of 82% and 95% respectively. This model was cross validated as described above, resulting in an accuracy of 80% for the train dataset (n=54), and 91% for the test dataset (n=35).

These results clearly demonstrate the benefit of including multiple plasma biomarkers to increase the diagnostic potential for mCRC. Using the three models described, the AUC was increased compared to any of the proteins alone. Furthermore, sensitivity and specificity were also increased.

Model Variables	Estimate	p value
APOA1 (mg/ml)	-0.069	0.043 (*)
IL-2 (pg/ml)	-0.029	0.006 (**)
Tenascin-C (ng/ml)	0.270	0.004 (**)
TIMP1 (ng/ml)	0.009	0.029 (*)

Table 3.5 Output of logistic regression model for identifying mCRC patients including APOA1, IL-2, Tenascin-C, and TIMP-1 as variables.

A logistic regression was performed using presence of mCRC as a binary variable for mCRC patients (n=50) and healthy controls (n=39), with measured values of APOA1, IL-2, Tenascin-C, and TIMP-1 as continuous variables. Estimate and p value as shown.

Model Variables	Estimate	p value
MMP-13 (pg/ml)	-0.007	0.009 (**)
Tenascin-C (ng/ml)	0.249	0.002 (**)
TIMP-1 (ng/ml)	0.014	0.002 (**)

Table 3.6 Output of logistic regression model for identifying mCRC patients including MMP-13, Tenascin-C, and TIMP-1 as variables.

A logistic regression was performed using presence of mCRC as a binary variable for mCRC patients (n=50) and healthy controls (n=39), with measured values of MMP-13, Tenascin-C, and TIMP-1 as continuous variables. Estimate and p value as shown.

Model Variables	Estimate	p value
IL-2 (pg/ml)	-0.034	0.001 (***)
Tenascin-C (ng/ml)	0.411	5.97E-06 (****)
TIM-3 (ng/ml)	0.346	0.001 (***)

Table 3.7 Output of logistic regression model for identifying mCRC patients including IL-2, Tenascin-C, and TIM-3 as variables.

A logistic regression was performed using presence of mCRC as a binary variable for mCRC patients (n=50) and healthy controls (n=39), with measured values of IL-2, Tenascin-C and TIM-3 as continuous variables. Estimate and p value as shown.

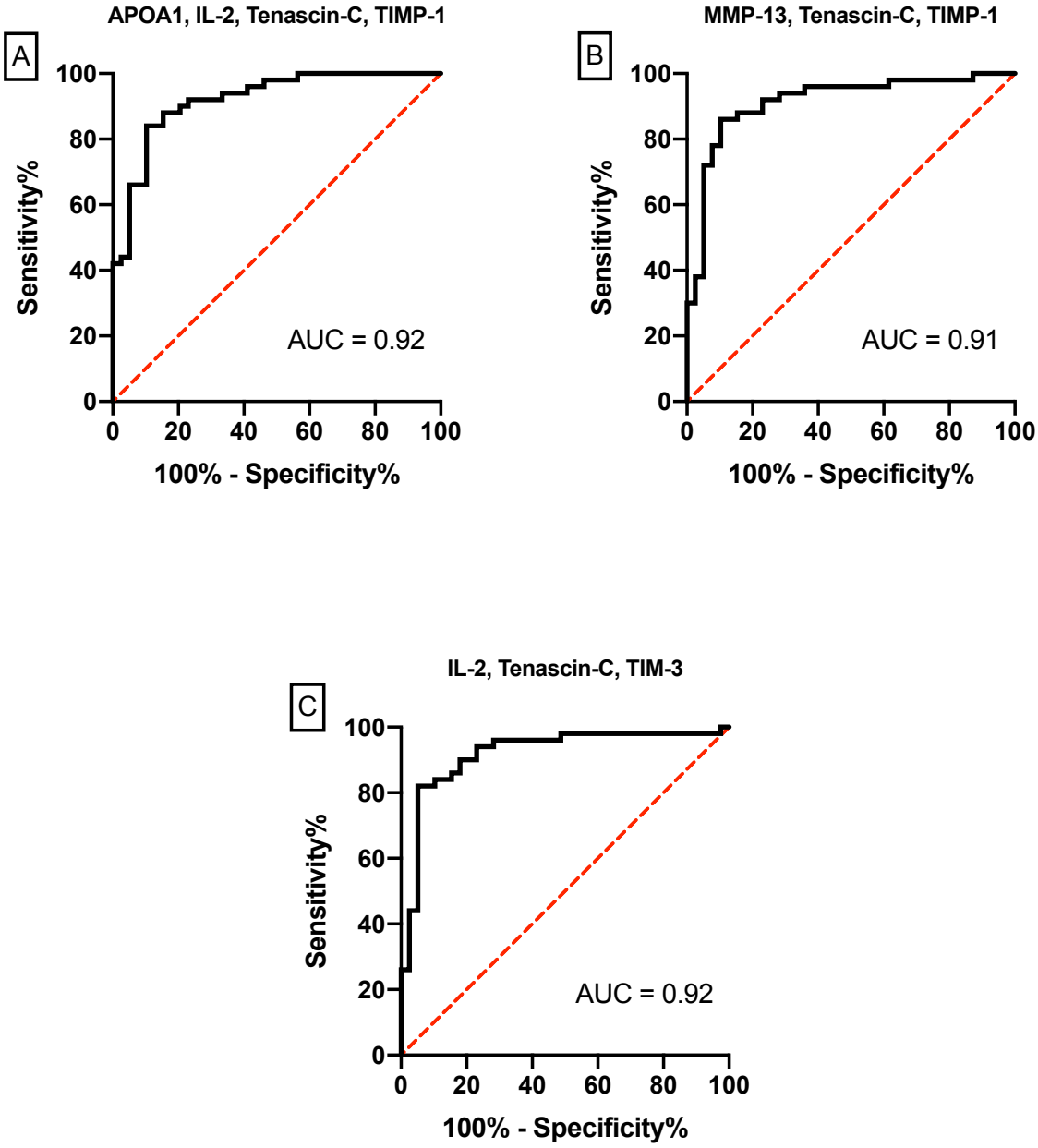


Figure 3.11 (A-C) ROC curves of logistic regression models for identifying mCRC patients.

ROC curves were plotted using logistic predictor scores generated from each model, for mCRC patients (n=50) and healthy controls (n=39). Variables included in the model are as described; A) APOA1, IL-2, Tenascin-C, TIMP-1 B) MMP-13, Tenascin-C, TIMP-1 C) IL-2, Tenascin-C, TIM-3.

It has been shown that MMP-13, PIGF-1, RANTES, and TIMP-1 are able to cluster earlier-stage patients and healthy controls with a sensitivity of 92% and a specificity of 64% (Figure 3.7). Furthermore, using ROC curve analysis, it was shown that these plasma proteins have individual diagnostic capacity for earlier-stage disease. A logistic regression model was built to identify earlier-stage disease compared to controls using these 4 proteins. When combined, proteins showed no significance. A backwards selection approach was employed to refine the model and showed that RANTES alone had the best capacity for identifying earlier-stage CRC patients. This has previously been shown in Table 3.3 and Figure 3.9C.

Previous analyses in this thesis have shown the ability of plasma proteins to distinguish between mCRC patients and earlier-stage CRC patients. A logistic regression model was built using the plasma proteins known to be significantly altered between these groups: APOA1, BDNF, IL-2, MMP-3, MMP-9, Tenascin-C, and TIMP-1. A backwards selection approach was employed, and the resulting model included APOA1, BDNF, and TIMP-1. Using the logistic predictor values from this model, ROC curve analysis was performed, resulting in an AUC of 0.90, $p<0.0001$ (Figure 3.12). Using Youden's Index, this model allowed for a sensitivity of 92% and a specificity of 75%, an overall improvement from the values for each protein alone. Although APOA1 and TIMP-1 did not show statistically significant estimates or odds ratios within the model (Table 3.8), they are trending towards significance. This may be a result of the comparatively small number of earlier-stage CRC patients used to build the model.

Model Variables	Estimate	p value
APOA1 (mg/ml)	-0.068	0.063
BDNF (pg/ml)	-0.012	0.020 (*)
TIMP1 (ng/ml)	0.015	0.080

Table 3.8 Output of logistic regression model for identification of mCRC patients compared to earlier-stage CRC patients including APOA1, BDNF, and TIMP1.

A logistic regression was performed using presence of mCRC as a binary variable for mCRC patients (n=50) and earlier-stage CRC patients (n=12), with measured values of APOA1, BDNF, and TIMP-1 as continuous variables. Estimate and p value as shown.

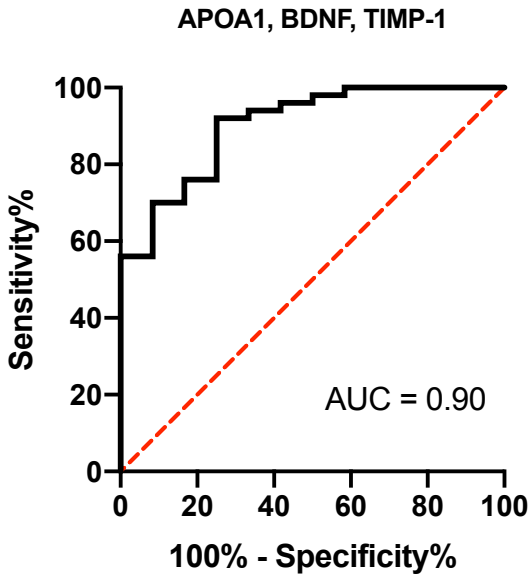


Figure 3.12 ROC curve of logistic regression model for identifying mCRC patients compared to earlier-stage CRC patients.

ROC curve was plotted using logistic predictor values generated from the model, for mCRC patients (n=50) and earlier-stage CRC patients (n=12). Variables included in the models are: APOA1, BDNF and TIMP-1.

3.3 Discussion

There is an unmet need for quick, easy, and reliable biomarkers for the identification of patients with CRC. It was hypothesised that a panel of plasma proteins, selected due to previously described associations with cancer and/or immunity, would be able to differentiate between healthy controls, patients with advanced mCRC, and patients with earlier-stage disease. This hypothesis was addressed using Luminex Multiplex technology to measure the levels of a panel of 31 plasma proteins in these groups. Acquiring the biological material to perform these assays is non-invasive, requiring only a small volume of blood plasma. It is time-efficient and enables the researcher to investigate multiple proteins in parallel, further reducing the sample volume required. The results described in this Chapter show that the levels of many plasma matrix and immune proteins are significantly altered between individuals with mCRC, those with earlier-stage CRC, and healthy controls. These could be attractive candidates for CRC diagnostic testing.

The results presented here show that the plasma levels of several matrix proteins, including MMPs, TIMPs, and Tenascin-C, are altered in patients with CRC compared to controls. These results are strongly supported by previous studies (Hurst *et al.* 2007; Väyrynen *et al.* 2012; Niewiarowska *et al.* 2014; Klupp *et al.* 2016). Increases in levels of many of these proteins, including MMP-2, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, and TIMP-1, have been associated with CRC progression and stage, and often correlate with reduced survival outcomes (Maurel *et al*. 2007; Kostova *et al*. 2012; Böckelman *et al*. 2018; Sirniö *et al*. 2018). Most of these previous studies have been performed using serum rather than plasma. The levels of several MMPs and TIMPs (including MMP-1, -2, -7, -8, -9, -13, and TIMP-1) are increased in serum compared to plasma (Mannello 2008; Jonsson *et al*. 2016). Although the exact reason for this is not fully understood, it is thought to be a result of protease release induced by the collection process; plasma is obtained using anticoagulant-treated tubes, whereas serum is collected by allowing blood to clot. It is possible that MMPs/TIMPs are released from platelets and leukocytes upon aggregation, and that the use of anticoagulants limits this (Sawicki *et al*. 1997). This may have led to inconsistencies in previous studies when assessing these proteins as diagnostic markers. A few studies, including the work described in this Chapter, have, however, measured matrix proteins in the plasma of CRC patients, and found similar results to those performed in serum; that they are often increased, frequently relating to worse survival (Holten-Andersen *et al*. 1999; Langenskiöld *et al*. 2005). Most of the previous studies investigating MMPs/TIMPs/Tenascin-C as potential diagnostic markers for CRC looked at 1-3

proteins. Moreover, such studies have not employed the use of logistic regression and hierarchical clustering to assess combinations of markers. The results described in this Chapter therefore represent a more comprehensive investigation of the potential of these proteins as markers of CRC patient identification, using Luminex Multiplex technology.

The interaction of the immune microenvironment and cancer has been widely investigated and has led to extensive therapeutic exploitation in recent years. The work described in this thesis has demonstrated that the circulating plasma levels of many immune-related proteins are significantly altered in patients with CRC with and without metastasis compared to both each other, and healthy controls. These changes may reflect underlying immunological processes and changes associated with CRC development and progression. The levels of APOA1, BDNF, and IL-2 for example are shown to be significantly reduced between mCRC patients and controls. These changes are further evident between patients with and without metastasis. Previously it has been demonstrated that serum BDNF levels are decreased in patients with CRC compared to healthy controls, but not altered between patients with different Dukes' staging of disease (Brierley *et al*. 2013). APOA1 has also been previously described as decreased in the serum of CRC patients (Engwegen *et al*. 2006; Peltier *et al*. 2016). Furthermore, reduced serum levels have been shown to correlate with reduced survival, immune responses, systemic inflammation, and advanced tumour staging (Walter *et al*. 2012; Sirniö *et al*. 2017). Although these previous reports generally support the findings of this thesis, they were performed using serum as opposed to plasma. Moreover, this thesis has explored both their individual and combined potential as diagnostic markers.

The work described in this thesis also demonstrates that the levels of IL-2, IL-15, IL-21, IP-10, PIGF-1, and RANTES are altered between mCRC patients and healthy controls. Furthermore, RANTES and PIGF-1 are altered between early-stage CRC patients and controls. The cytokine/immune profile of CRC patients compared to controls has been previously investigated in a mixture of plasma and serum samples (Kantola *et al*. 2012; Gunawardene *et al*. 2019; Yamaguchi *et al*. 2019). In such studies, the levels of IL-2 and IL-15 were unable to be measured due to technical difficulties. Findings based on IP-10 levels between CRC patients and controls were inconsistent between these studies, and those for RANTES opposed the results of this thesis. These previous reports do not strongly support the findings described in this Chapter, but instead suggest that changes in these proteins are complex, and their relationship to CRC is not fully understood.

This Chapter demonstrates global serological changes associated with CRC, which are further emphasised in metastatic disease. Many plasma proteins were found to be significantly altered in mCRC and earlier-stage CRC patients compared to healthy controls. Similarly, plasma proteins were also identified as altered between patients based on the presence/absence of metastatic disease. These striking observations suggest a role of these proteins in CRC development, progression, and metastasis, moreover, the identified proteins and their associated pathways may represent valid therapeutic targets for CRC treatment.

The usefulness of the identified proteins as diagnostic markers of earlier-stage CRC and mCRC was investigated and has identified clear candidates. RANTES and TIMP-1 showed good diagnostic accuracy for earlier-stage CRC compared to controls. Furthermore, APOA1, BDNF, IL-2, MMP-3, Tenascin-C, and TIMP-1, showed a range of diagnostic accuracy for mCRC patients compared to earlier-stage patients. APOA1, BDNF, IL-2, IL-21, MMP-13, Tenascin-C, and TIMP-1 also showed a range of diagnostic accuracy for mCRC patients compared to healthy controls. These findings support previously described candidate markers and help to identify new proteins of interest.

TIMP-1 serum levels have previously been described as increased in CRC patients compared to healthy controls, and as increased in advanced CRC. Furthermore, it has been implicated as a potential CRC biomarker in a number of studies, with sensitivity and specificity values similar to the findings of this thesis (Holten-Andersen *et al.* 1999; Niewiarowska *et al.* 2014; Böckelman *et al.* 2018; Meng *et al.* 2018). Large spliced variant Tenascin-C has also been described as a biomarker of CRC, and found to increase with tumour progression (Takeda *et al*. 2007). These reports strongly support the findings of this thesis that TIMP-1 and Tenascin-C represent potential diagnostic markers of CRC, which may also provide information on disease stage. Previous studies have also suggested that although altered in CRC compared to controls, BDNF shows poor sensitivity and specificity as a biomarker (Brierley *et al*. 2013). Conversely, the result of this thesis identifies BDNF as a potential CRC biomarker. Importantly, the previous study used serum BDNF, whereas the results described in this Chapter use plasma.

It is clear from the above analyses that several matrix and immune plasma proteins may provide useful clinical information for identifying earlier-stage CRC patients and mCRC patients compared to healthy controls. Furthermore, in known CRC patients, these proteins also have the ability to identify those individuals with metastatic disease. These identified proteins perform at a range of sensitivities and specificities and

represent potential biomarkers for CRC. As the group size of earlier-stage CRC patients is relatively small (n=12-14), these experiments should be repeated on a larger cohort to confirm these preliminary findings.

Diagnostic markers often fail in clinical settings due to their lack of sensitivity and/or specificity. From the results shown in this Chapter, it is clear that no one plasma protein is able to identify the presence of early-stage CRC or mCRC with 100% accuracy. This would stand to reason, given that these proteins are relevant to a number of normal, disease-independent, biological mechanisms. It could be argued that all biological protein measurements may have limited sensitivity and/or specificity, for this reason. A key finding of this thesis is the improved diagnostic capacity of plasma protein markers when in combination. This would be a useful concept to employ when establishing new diagnostic tests for a range of diseases. Hierarchical cluster analysis and logistic regression have demonstrated this concept clearly throughout this Chapter.

Using APOA1, IL-2, Tenascin-C, and TIMP1 as variables, logistic regression was able to separate mCRC patients and healthy controls with a sensitivity of 84% and a specificity of 90%. This combination was more sensitive and specific than each protein was alone. Similarly, hierarchical clustering strongly supported this finding. Hierarchical clustering and logistic regression also provided some evidence that combinations of plasma proteins are able to differentiate between earlier-stage CRC patients and healthy controls, and between CRC patients with and without metastasis.

Multiple potential biomarkers of earlier-stage CRC, and mCRC have been identified. These proteins have the potential to act as early warning signs of CRC and metastatic disease, and this should be explored in a larger cohort of CRC patients (stage I-IV) and healthy controls. This would allow the logistic regression models described to be refined and validated. The diagnosis of CRC is invasive, and early symptoms often go unreported. A blood test to help diagnosis could be usefully employed, in addition to other testing, to screen high-risk patient groups, for example those currently targeted by Bowel Screening Wales, patients with family history of CRC, and patients with conditions such as polyps/ulcerative colitis/Crohn's. If levels of the identified proteins were increased in these patients to a level seen in CRC, then they could be referred for more detailed investigations. Measurements of the identified proteins of interest could be taken every 2 years, as per current screening methods for Bowel Screening Wales, or during routine follow up appointments for other risk groups. This would allow the models described in this thesis to be improved, and findings could be directly compared to current screening methods.

The role of matrix and immune proteins in identifying earlier-stage CRC, and the presence of distant metastasis in CRC patients warrants further investigation. The group size of earlier-stage patients was small (n=14), therefore it would be important to confirm the findings described here in a larger cohort of CRC patients with stage I-III disease. Not only would this allow an investigation into their potential for identifying metastatic disease in known CRC patients, but it would also be possible to assess their potential in identifying early-stage disease by comparing to healthy controls. Clearly identifying metastasis can be difficult, particularly when lesions are small. Moreover, survival of patients with metastatic disease is significantly reduced compared to earlier-stage CRC. It would therefore be very interesting to assess the relationship between levels of the identified markers of mCRC, for example TIMP-1 and Tenascin-C, and size and number of metastatic lesions. This could be done retrospectively using the cohort of TaCTiCC patients by assessing metastatic burden, however given the advanced nature of this cohort, it should also be performed on additional mCRC patients. If metastasis could be identified earlier then it could improve survival outcomes and inform treatment strategy. It would also be interesting to assess the potential of these proteins as markers for identifying CRC patients who relapse. One could measure these markers in a group of CRC patients in remission during routine follow-up appointments. These levels could be monitored across time and if increased to the levels seen in active disease, then they could be recommended for more detailed investigations. The benefit of this could easily be assessed afterwards based on clinical diagnosis of patient relapse. Furthermore, it would be interesting to assess the levels of these proteins both pre- and post-resection of primary tumour and/or metastatic lesion, and during subsequent treatment. It could then be established whether levels return to those seen in healthy controls. This would further assess their usefulness for identifying patients at risk of relapse.

By comparing the levels of matrix/immune proteins in individuals with and without cancer, their importance in CRC has been validated. These altered proteins may play a role in disease development, progression, and metastasis. Moreover, many of them represent potential diagnostic biomarkers for the identification of early-stage and late-stage CRC. Importantly, when considered together, the diagnostic potential of these proteins is improved. This should be a consideration for all future biomarker development.

3.4 Key Findings

1. Luminex Multiplex represents a time-efficient and non-invasive method of measuring circulating plasma proteins in patients and controls, requiring very little biological material.

2. The plasma levels of several matrix and immune proteins are significantly altered between patients with earlier-stage CRC patients (Stage I-III), mCRC patients (Stage IV), and healthy controls. These may represent important pathways for CRC development and progression.

3. Individually many of these plasma proteins show good ability to identify i) mCRC patients compared to healthy controls ii) earlier-stage CRC patients compared to healthy controls iii) mCRC patients compared to earlier-stage CRC patients. These identified plasma proteins are potential CRC biomarkers.

4. The combination of multiple plasma proteins increases their ability to identify i) mCRC patients compared to healthy controls ii) earlier-stage CRC patients compared to healthy controls iii) mCRC patients compared to earlier-stage CRC patients. A combinatory approach may improve sensitivity and specificity of CRC biomarkers.

Group	Protein(s)	Test	AUC	Sensitivity (%)	Specificity (%)
Healthy Control & Early CRC					
Best Individual Protein	RANTES	Individual ROC Curve	0.71	83	64
Best Combination	MMP-13 PIGF-1 RANTES TIMP-1	Hierarchical Clustering	-	92	64
Healthy Control & mCRC					
Best Individual Protein	Tenascin-C	Individual ROC Curve	0.87	75	90
Best Combination	APOA1 IL-2 Tenascin-C TIMP-1	Logistic Regression	0.92	84	90

Early CRC & mCRC					
Best Individual Protein	Tenascin-C	Individual ROC Curve	0.83	65	93
Best Combination	APOA1 BDNF TIMP-1	Logistic Regression	0.90	88	75

Table 3.9 Overview of key proteins identified for differentiation of healthy controls, earlier-stage CRC patients, and mCRC patients.

This table describes the key proteins identified in this Chapter able to differentiate between i) healthy controls and earlier-stage CRC patients ii) healthy controls and mCRC patients iii) earlier-stage CRC patients and mCRC patients.

Chapter 4 Identification of Biomarkers of Response to Immunotherapeutic Cyclophosphamide and TroVax in Patients with Advanced Metastatic CRC

4.1 Introduction

Although there have been significant advances in cancer immunotherapy, many patients are still not responding to these therapies, and we do not clearly understand why. Being able to identify patients who will benefit could increase the success of immunotherapy, allowing patients to receive the most appropriate treatments. Additionally, it could help us to further understand mechanisms of cancer progression and treatment resistance. It was hypothesised that blood and protein measurements before and during treatment would enable prediction of treatment response. This was investigated in the context of a recently completed clinical trial: TroVax and Cyclophosphamide Treatment in Colorectal Cancer (TaCTiCC).

TaCTiCC was undertaken within the Godkin/Gallimore group and measured the effect of MVA-5T4 cancer vaccine and/or CPM treatment on anti-tumour immune responses in a group of 52 end-stage mCRC patients (Scurr *et al.* 2017a; Scurr *et al.* 2017b). Patients received one of the following treatments; TroVax (17), CPM (9), TroVax and CPM (18), none (8). Immunological responses and improved survival outcomes were demonstrated in around 50% of patients and are summarized in Appendix 2. Throughout the trial multiple serological/immunological parameters were measured in order to monitor treatment effect, the accepted normal range of these measurements is detailed in Supplementary Table 3:

1. 5T4 Antibody Response
2. 5T4 Cultured T Cell Response
3. Alanine transaminase (ALT)
4. Basophils
5. Bilirubin
6. $CD3^{+}CD4^{+}$ cells
7. $CD3^{+}CD8^{+}$ cells
8. Eosinophils
9. Glucose
10. Haematocrit

11. Haemoglobin
12. Lymphocytes
13. Mean Corpuscular Haemoglobin (MCH)
14. Mean Corpuscular Volume (MCV)
15. Monocytes
16. MVA Antibody Response
17. Neutrophil: Lymphocyte Ratio (NLR)
18. Neutrophils
19. Platelets
20. Red Blood Cell Count
21. T_{reg} Number
22. T_{reg} proportion
23. White Blood Cell Count (WBC)

Additionally, using frozen plasma samples from these patients, the levels of 31 proteins were retrospectively measured across treatment by Luminex Multiplex. These analytes were selected due to their previously reported associations with cancer and/or tumour immunity (Supplementary Table 2) as discussed in Chapter 3. The work described in this Chapter investigates the usefulness of these measurements for predicting treatment response to CPM and TroVax.

4.2 Results

4.2.1 Comparison of Pre-Treatment Serological, Immunological and Plasma Protein Measurements Between Cyclophosphamide Responders and Non-Responders

The usefulness of pre-treatment serological, immunological and plasma protein markers for predicting response to CPM was assessed by comparing levels between responders and non-responders in all TaCTiCC patients receiving CPM. Throughout the TaCTiCC clinical trial response to CPM was defined in several ways:

1. Decrease in T_{reg} number (>39.4%).
2. Increase in cultured T cell response to 5T4.
3. Patients who fit both of these criteria.

The cut-off percentage decrease in T_{reg} number between responders and non-responders was set at the upper 95% confidence interval of all values measured (39.4%). This resulted in clear responder and non-responder grouping, which translated into survival differences. Responders and non-responders were also grouped by increase in cultured T cell response to 5T4; responders more than doubled their cultured T cell response to 5T4 at any treatment day during TaCTiCC. Positive responses were also identified as having at least 20 spot forming cells per 10^5 cultured cells. These cut-off parameters are further described in (Scurr et al. 2017a).

When patients were split into CPM responders/non-responders based on decrease in T_{reg} number, none of the 31 pre-treatment plasma proteins were significantly different. There was, however, a significantly higher pre-treatment T_{reg} number in non-responders compared to responders (Figure 4.1).

Next, patients were split into CPM responders/non-responders based on IFNγ response to 5T4. None of the measured serological/immunological parameters were significantly altered between these groups. Of the 31 measured plasma proteins, TIMP-1 was significantly higher in patients who did not respond to treatment (Figure 4.2).

Patients were also split into responders/non-responders based on both magnitude of T_{reg} depletion and IFNγ response to 5T4. The levels of basophils, $CD3^+CD4^+$ cells, T_{reg} cells, and white blood cells (WBCs) were significantly increased in CPM non-responders compared to responders (Figure 4.3 A-D). Similarly, plasma protein levels of MMP-3 and

TIMP-1 were significantly higher, and the level of Tenascin-C was generally increased, in CPM non-responders (Figure 4.3 E-G). As responders to both criteria could be reasonably regarded as the most immunologically responsive patients to CPM treatment, this was considered the response criteria for the remainder of this Chapter.

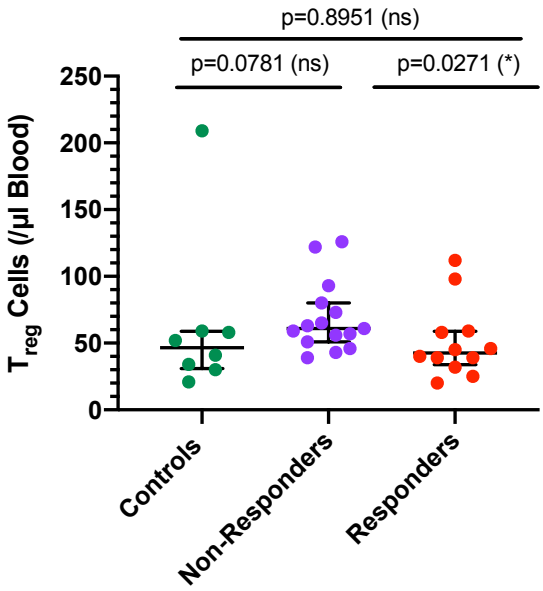


Figure 4.1 The number of T_{reg} cells are significantly higher in mCRC patients who do not respond to CPM treatment.

CPM-treated patients were split into those who responded to therapy (n=12), those who did not (n=15), based on magnitude of T_{reg} depletion. Untreated control patients are also shown (n=8). Groups were compared using Mann-Whitney U test. Median and interquartile range shown, and asterisk indications of significance are per methods.

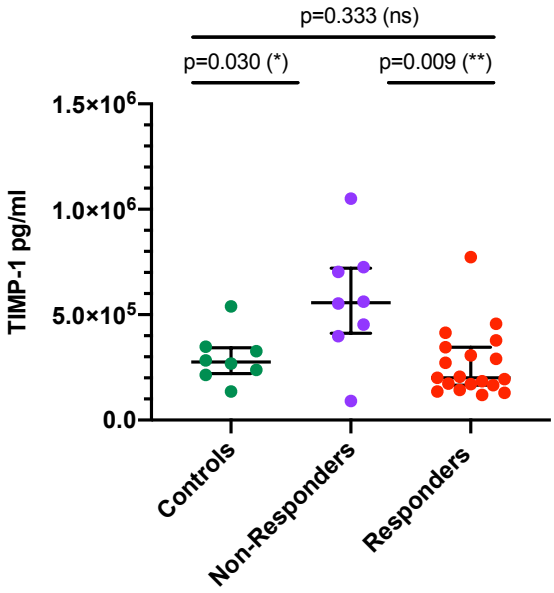
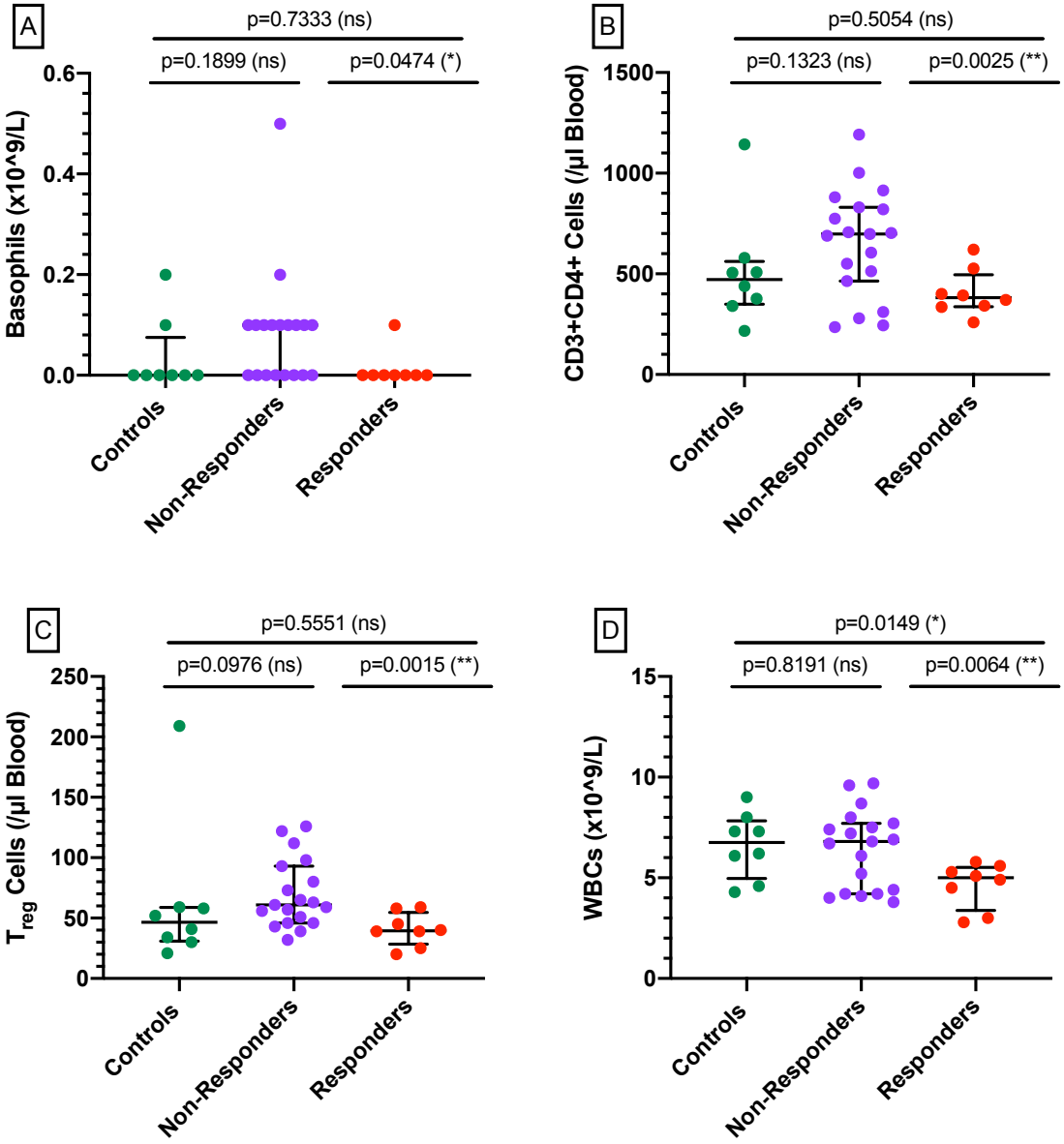


Figure 4.2 The level of plasma TIMP-1 is significantly higher in mCRC patients who do not respond to CPM treatment.

CPM-treated patients were split into those who responded to therapy (n=19), those who did not (n=8), based on magnitude of IFNγ response to 5T4. Untreated control patients are also shown (n=8). Groups were compared using Mann-Whitney U test. Median and interquartile range shown, and asterisk indications of significance are as per methods.


A
p=0.7333 (ns)
p=0.1899 (ns)
p=0.0474 (*)
Basophils (x10^9/L)
0.6
0.4
0.2
0.0
Controls
Non-Responders
Responders
B
p=0.5054 (ns)
p=0.1323 (ns)
p=0.0025 (**)
CD3+CD4+ Cells (/µl Blood)
1500
1000
500
0
Controls
Non-Responders
Responders
C
p=0.5551 (ns)
p=0.0976 (ns)
p=0.0015 (**)
T reg Cells (/µl Blood)
250
200
150
100
50
0
Controls
Non-Responders
Responders
D
p=0.0149 (*)
p=0.8191 (ns)
p=0.0064 (**)
WBCs (x10^9/L)
15
10
5
0
Controls
Non-Responders
Responders

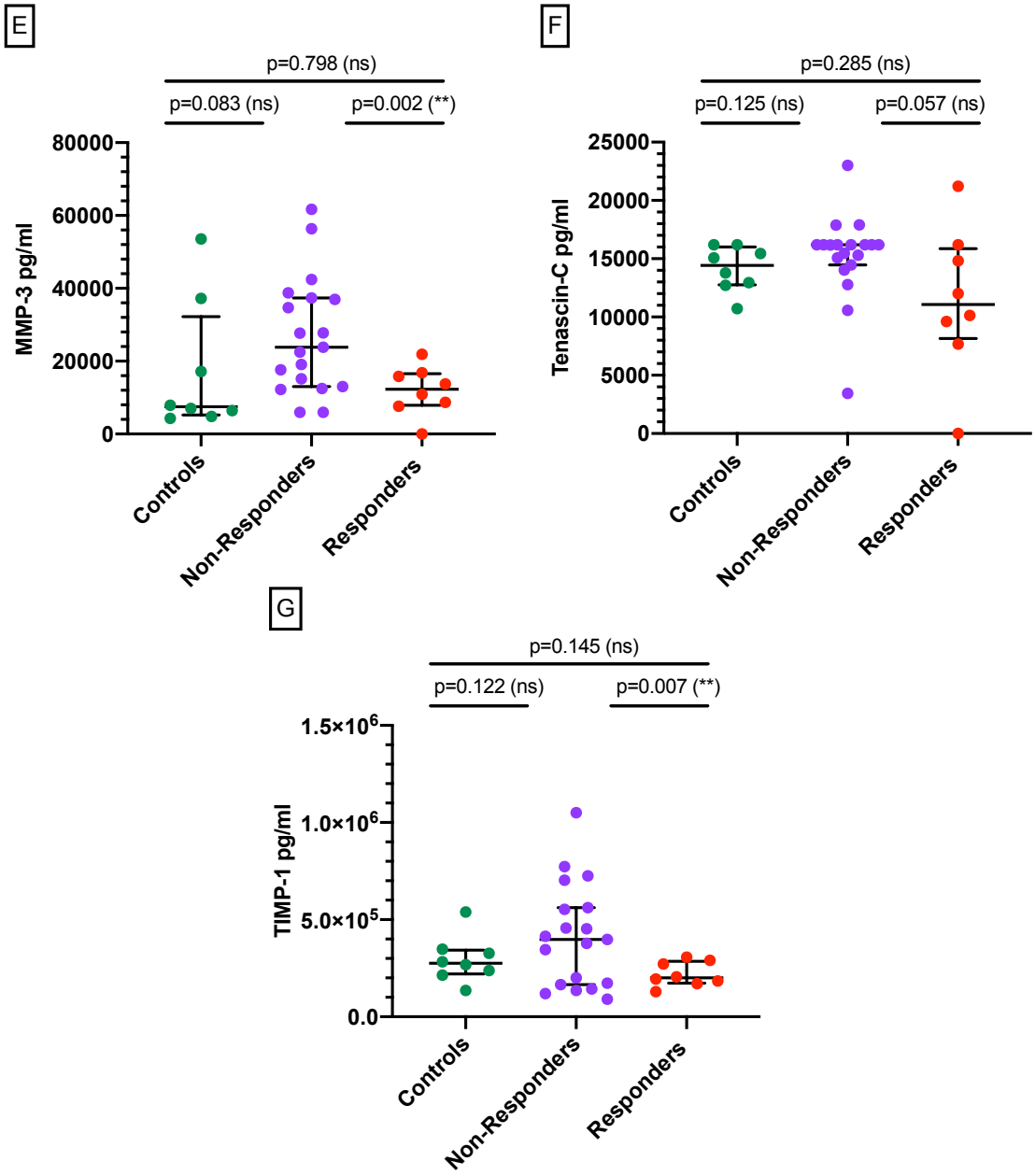


Figure 4.3 (A-G) Multiple immunological parameters and plasma matrix protein measurements are significantly higher in mCRC patients who do not respond to CPM treatment.

CPM-treated patients were split into those who responded to therapy (n=8), those who did not (n=19), based on magnitude of T_{reg} depletion and IFNγ response to 5T4. Untreated control patients are also shown (n=8). Groups were compared using Mann-Whitney U test. Median and interquartile range shown, and asterisk indications of significance are as per methods.

4.2.2 Association of Pre-Treatment Biomarkers of Response to Cyclophosphamide with Overall and Progression-Free Survival

TaCTiCC patients who immunologically responded to therapy demonstrated improved survival outcomes. It was therefore hypothesised that the biomarkers of CPM response identified in Section 4.2.1 would also predict survival outcomes in this patient cohort:

1. Basophils
2. $CD3^+CD4^+$ cells
3. T_{reg} cells
4. WBCs
5. MMP-3
6. Tenascin-C
7. TIMP-1

This was investigated by log-rank test and displayed using Kaplan-Meier plots, for both overall and progression-free survival. As these variables are continuous in nature, they were first split into categorical variables. Plasma protein markers, MMP-3, Tenascin-C, and TIMP-1, were converted into two groups using the uppermost level measured for healthy individuals as the cut-off value between high and low. The levels in healthy donors are described in Chapter 3. Basophil and WBC counts were split into high and low using the standard accepted healthy values used by the NHS as the cut-off value (detailed in Supplementary Table 3). T_{reg} and $CD3^+CD4^+$ cell numbers were split based on mean, median, and the 75% quartile of the TaCTiCC cohort.

High plasma MMP-3 significantly associated with reduced progression-free survival (PFS) when considering patients receiving CPM as part of their treatment (Figure 4.4B), however not when considering the entire TaCTiCC cohort (Figure 4.4A). Plasma Tenascin-C did not significantly associate with either overall or progression-free survival within the entire TaCTiCC cohort, or within CPM-treated patients (data not shown). High plasma TIMP-1 significantly associated with decreased overall survival both in the entire cohort, and in CPM-treated patients (Figure 4.5 A and B). There was also a trend towards high TIMP-1 associating with reduced PFS (Figure 4.5 C and D).

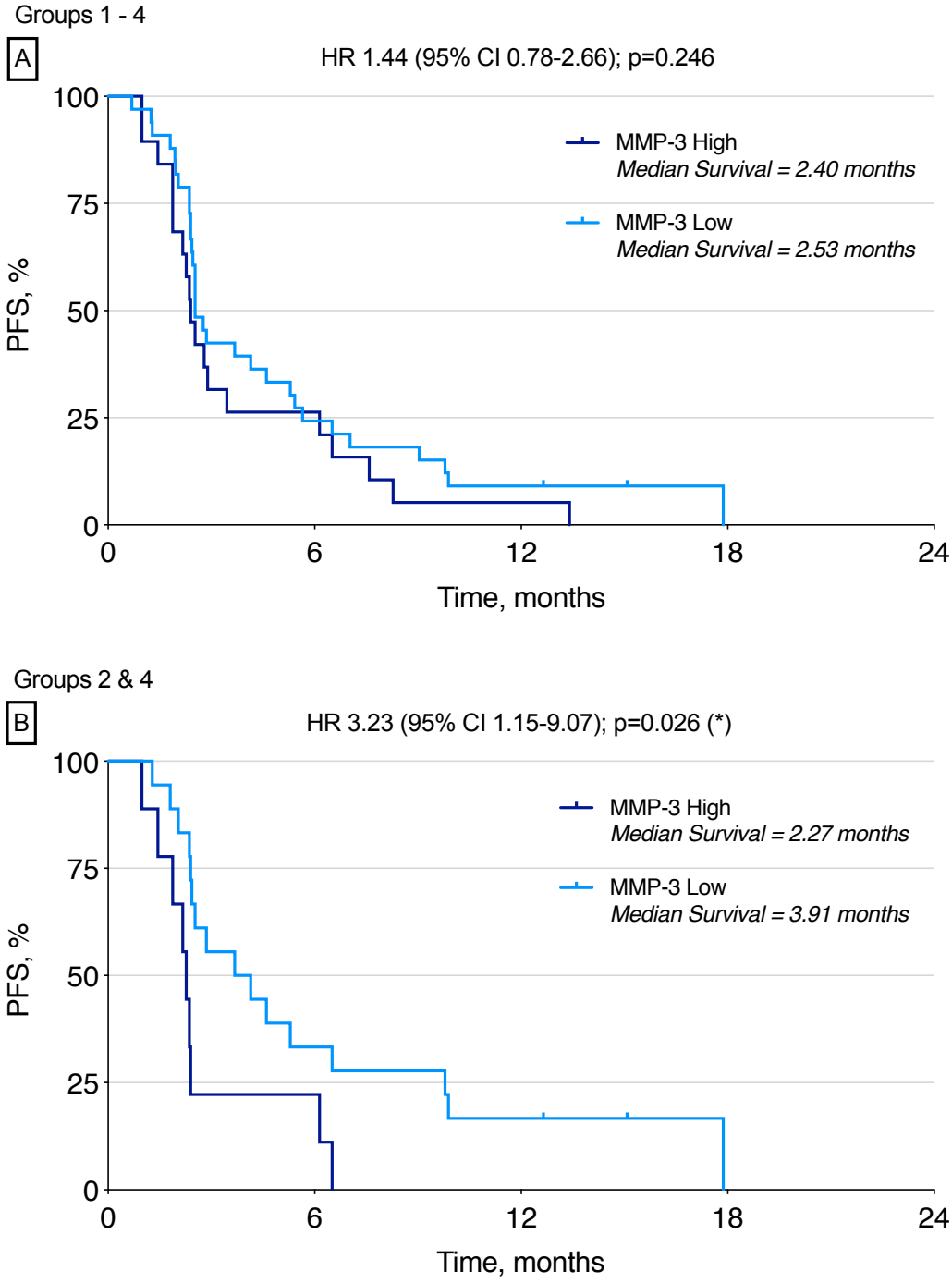
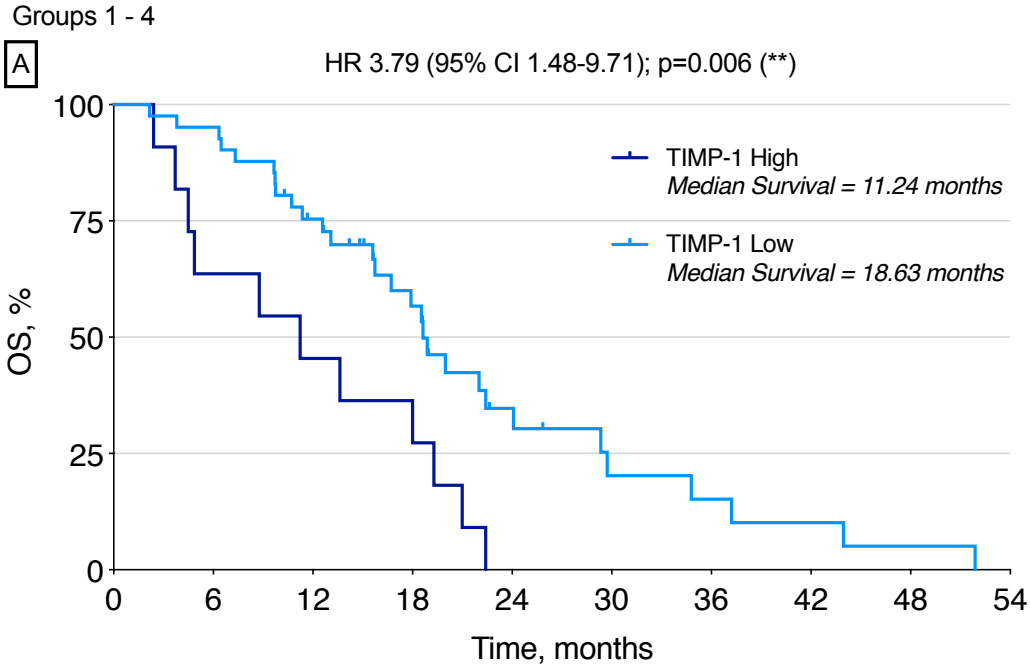
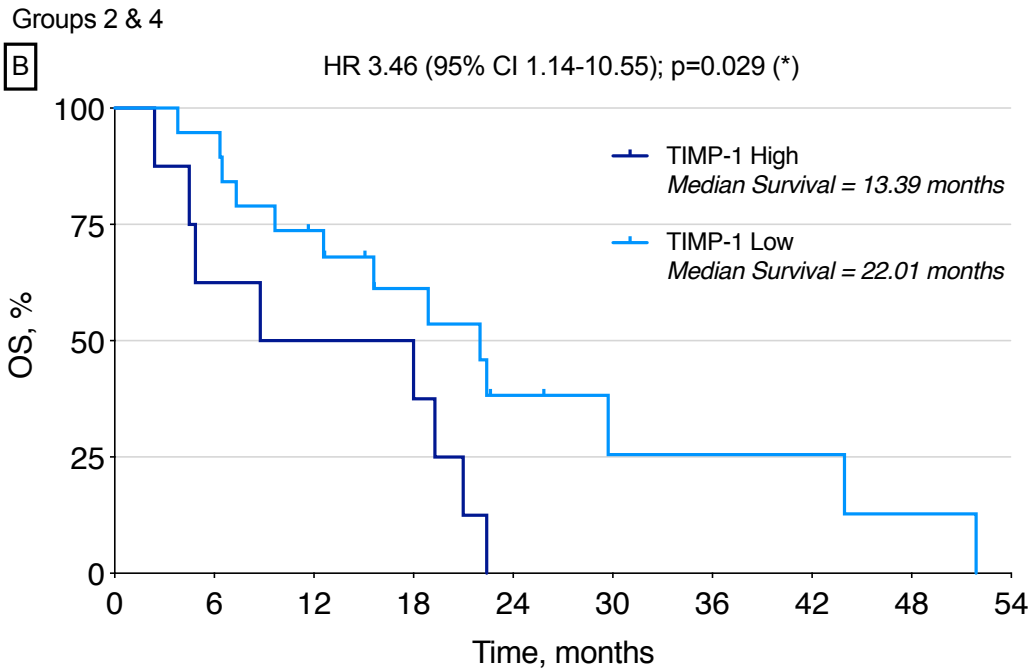
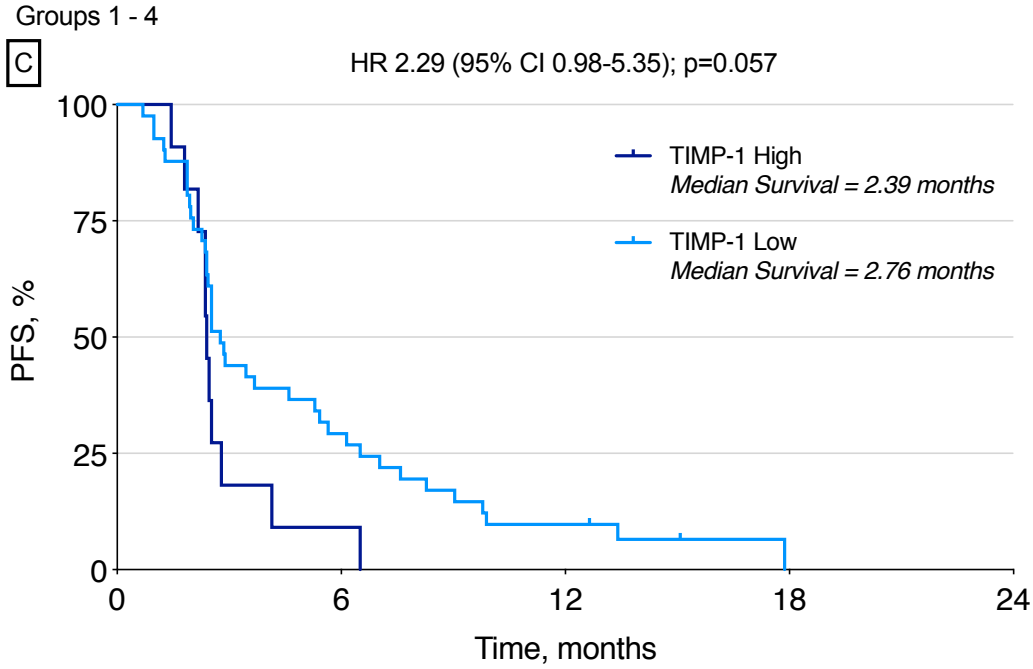


Figure 4.4 (A-B) Kaplan-Meier survival curves showing progression-free survival of TaCTiCC patients stratified by high and low MMP-3.

mCRC TaCTiCC patients were stratified by high and low MMP-3 using the uppermost value for healthy donors as a cut-off value. Progression-free survival was compared between high and low MMP-3 groups for the entire TaCTiCC cohort (A, n=52), or for CPM-treated patients (B, n=27). Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown.


Groups 1 - 4
A
HR 3.79 (95% CI 1.48-9.71); p=0.006 (**)
100
75
50
25
0
OS, %
TIMP-1 High
Median Survival = 11.24 months
TIMP-1 Low
Median Survival = 18.63 months
0
6
12
18
24
30
36
42
48
54
Time, months


Groups 2 & 4
B
HR 3.46 (95% CI 1.14-10.55); p=0.029 (*)
100
75
50
25
0
OS, %
TIMP-1 High
Median Survival = 13.39 months
TIMP-1 Low
Median Survival = 22.01 months
0
6
12
18
24
30
36
42
48
54
Time, months



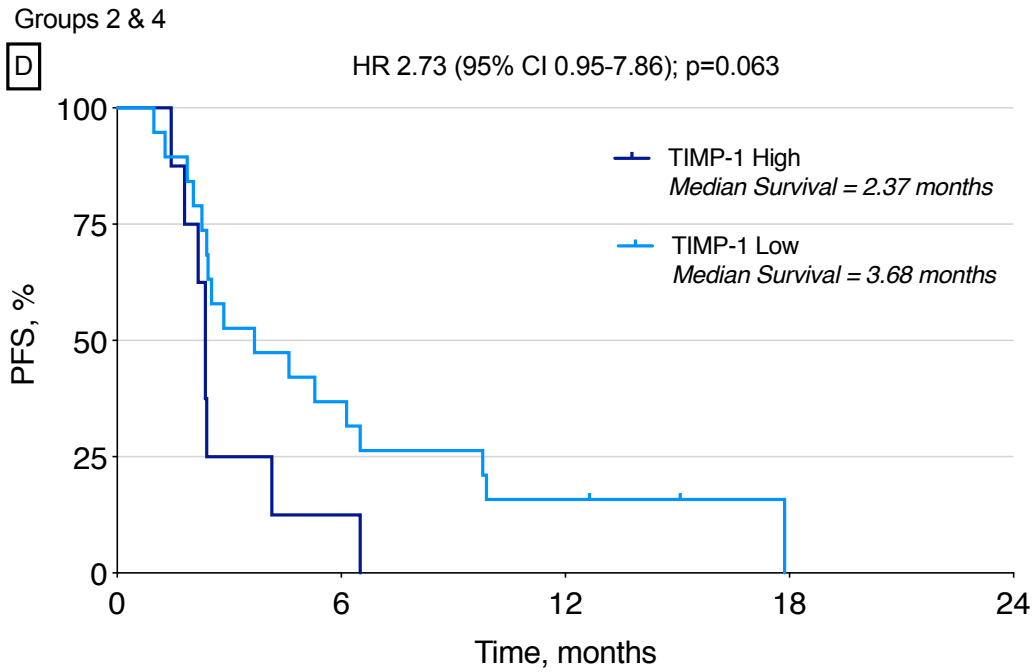
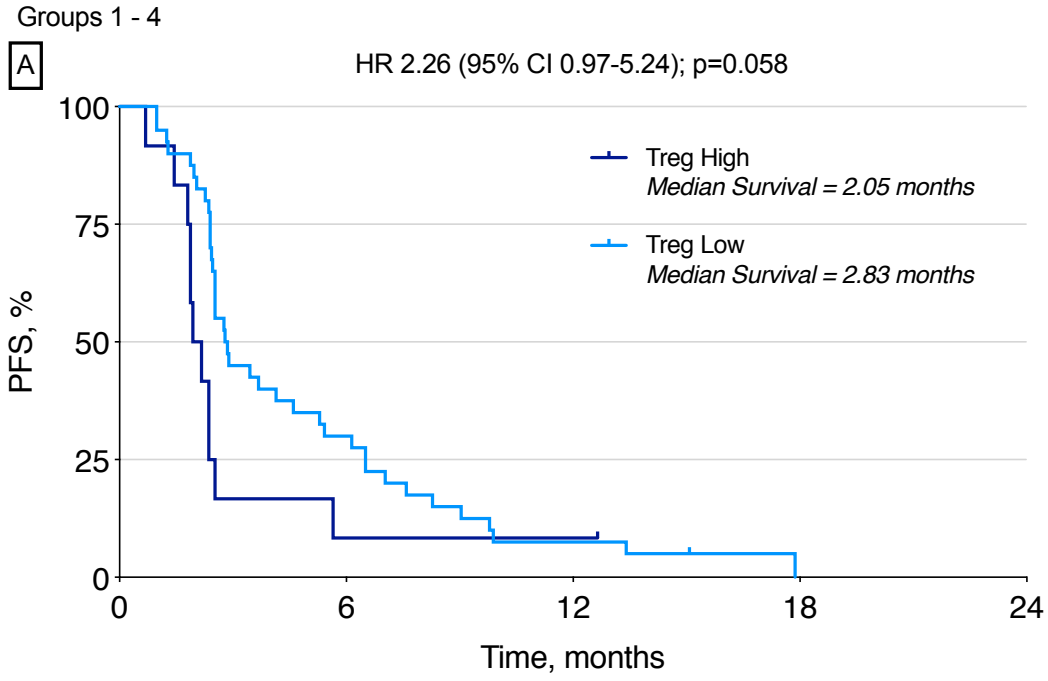


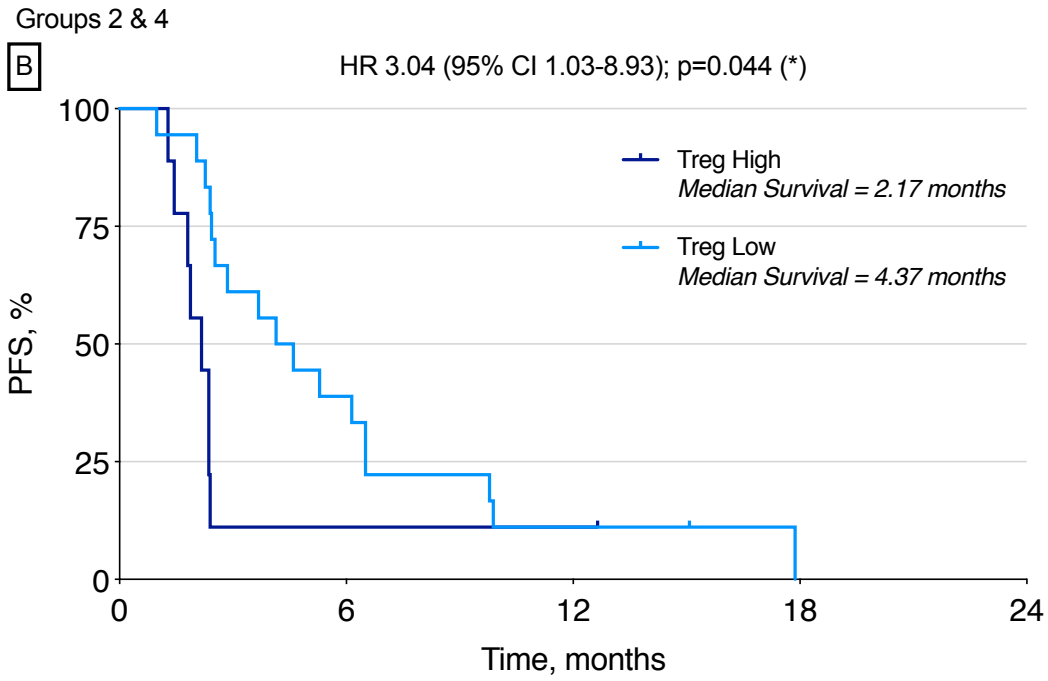
Figure 4.5 (A-D) Kaplan-Meier survival curves showing overall/progression-free survival of TaCTiCC patients stratified by high and low TIMP-1.

mCRC TaCTiCC patients were stratified by high and low TIMP-1 using the uppermost value for healthy donors as a cut-off value. Overall survival was compared between high and low TIMP-1 groups for the entire TaCTiCC cohort (A, n=52), and for CPM-treated patients (B, n=27). This was also performed for progression-free survival (C and D). Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown.

There were no significant associations between basophil or WBC number and overall or progression-free survival, either in the combined TaCTiCC cohort, or in CPM-treated patients (data not shown).

The number of T_{reg} and $CD3^+CD4^+$ cells were measured within the Godkin/Gallimore group, therefore were not directly compared to a healthy reference group. To convert these measurements into categorical variables, groups were split by mean, median, and 75% quartile. When the entire TaCTiCC cohort was split into T_{reg} high and low based on the 75% quartile, there was a trend towards reduced progression-free survival in the T_{reg} high group (Figure 4.6A). There was also significantly reduced progression-free survival in the T_{reg} high group when considering CPM-treated patients split by mean T_{reg} level (Figure 4.6B), and a trend towards reduced overall survival in the T_{reg} high group when CPM-treated patients were split based on the 75% quartile (Figure 4.6C). When split by 75% quartile, there was significantly reduced progression-free survival in the $CD3^+CD4^+$ high group, in both the combined TaCTiCC cohort, and in CPM-treated patients (Figure 4.7 A&B). There was no difference in overall survival between the CD3+CD4+ high and low groups, regardless of stratification method, therefore data is not shown.


Groups 1 - 4
A
HR 2.26 (95% CI 0.97-5.24); p=0.058
100
75
50
25
0
PFS, %
Treg High
Median Survival = 2.05 months
Treg Low
Median Survival = 2.83 months
0
6
12
18
24
Time, months


Groups 2 & 4
B
HR 3.04 (95% CI 1.03-8.93); p=0.044 (*)
100
75
50
25
0
PFS, %
Treg High
Median Survival = 2.17 months
Treg Low
Median Survival = 4.37 months
0
6
12
18
24
Time, months

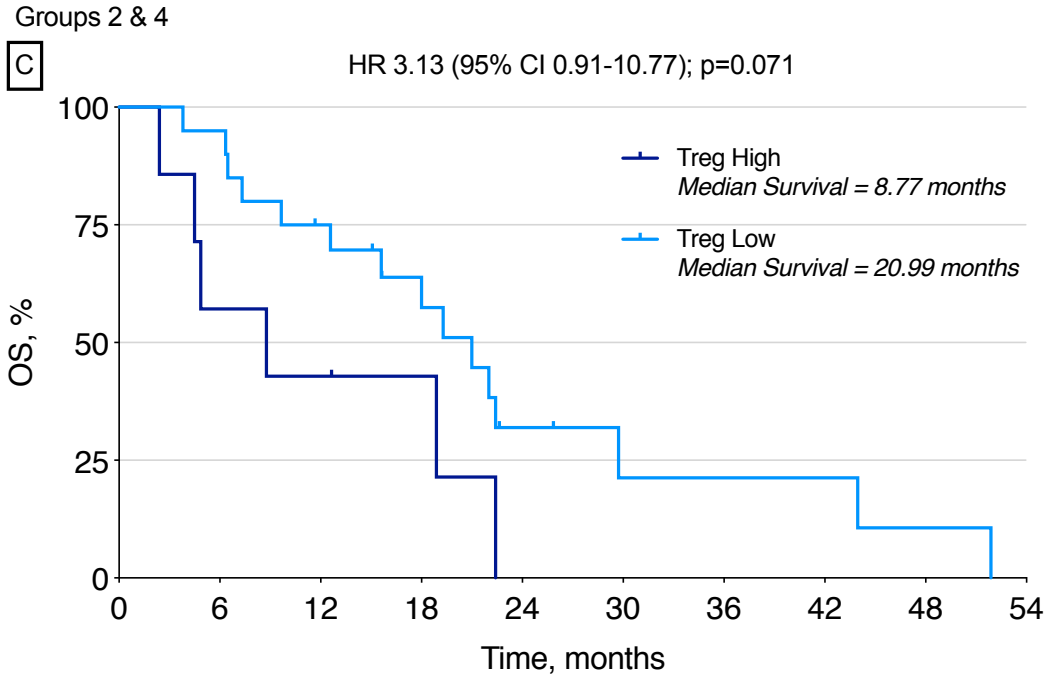


Figure 4.6 (A-C) Kaplan-Meier survival curves showing overall and progression-free survival of TaCTiCC patients stratified by high and low T_{reg} number.

mCRC TaCTiCC patients were stratified by high and low T_{reg} number using either 75% quartile (A and C) or mean (B) T_{reg} number. Progression-free survival was compared for the entire TaCTiCC cohort, or for CPM-treated patients (A and B, n=52 and n=27 respectively). Overall survival was compared for CPM-treated patients (C, n=27). Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown.

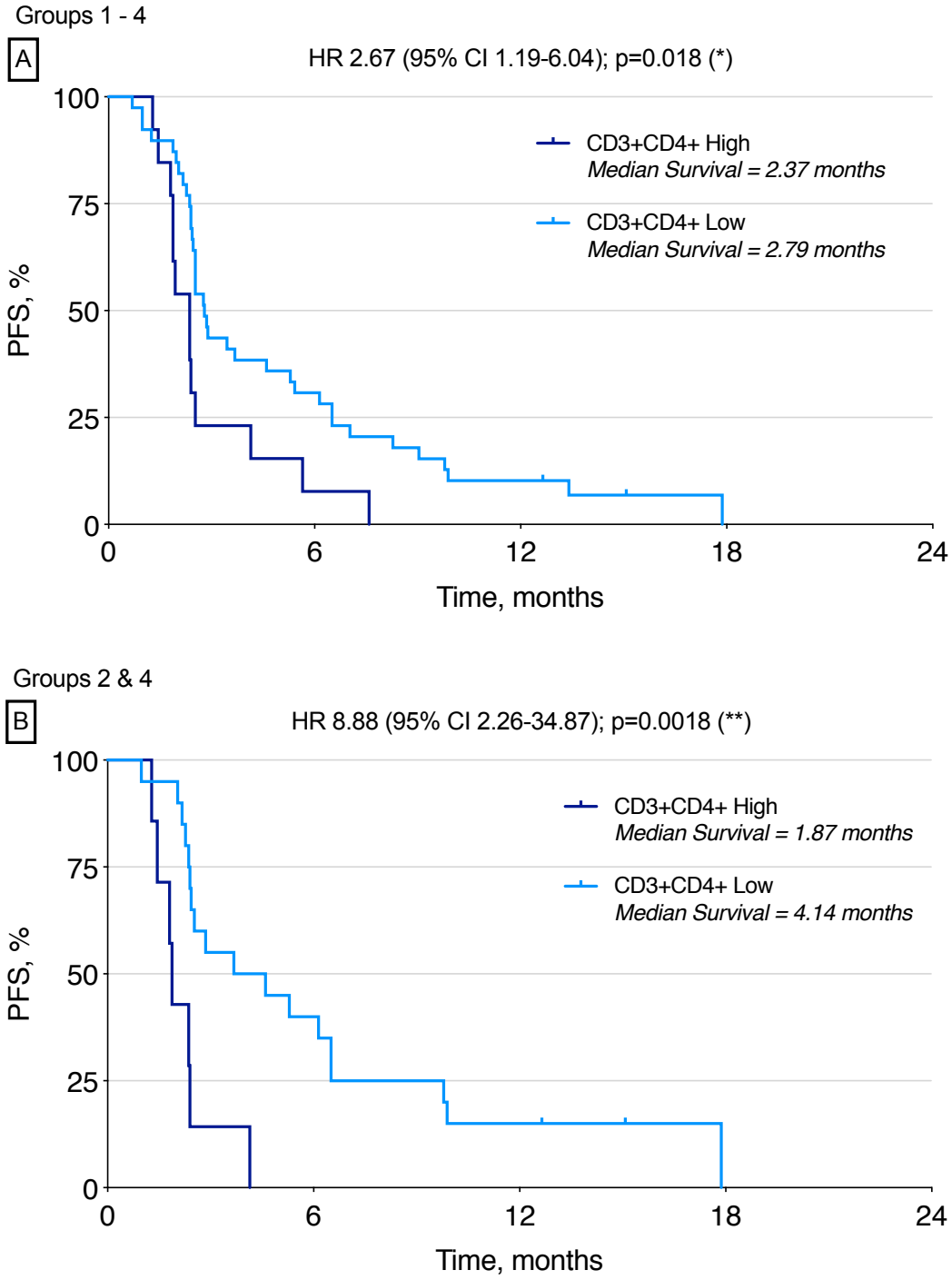


Figure 4.7 (A-B) Kaplan-Meier survival curves showing progression-free survival of TaCTiCC patients stratified by high and low $CD3^+CD4^+$ number. mCRC TaCTiCC patients were stratified by high and low $CD3^+CD4^+$ number using 75% quartile. Progression-free survival was compared for the entire TaCTiCC cohort, or for CPM-treated patients (A and B, n=52 and n=27 respectively). Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown. ***There was no observed difference in overall survival.***

4.2.3 Individual Pre-Treatment Immunological and Plasma Protein Measurements as Potential Diagnostic Markers to Identify Cyclophosphamide Responders

The results from Section 4.2.1 have clearly demonstrated that pre-treatment levels of several immunological parameters and matrix-associated proteins are significantly altered between individuals who respond to CPM immunotherapy, and those who do not. Many of these markers also associate with overall/progression-free survival, as shown in Section 4.2.2. It was therefore hypothesised that these measurements also have the capacity to predict treatment response. To assess how these measurements would individually perform in a diagnostic test for CPM response, receiver-operating characteristic (ROC) curve analysis was performed on each on the following measurements:

1. $CD3^+CD4^+$cell number
2. T_{reg} cell number
3. WBC number
4. Plasma MMP-3
5. Plasma TIMP-1

Basophil number, although significantly altered, had a high proportion of identical measurements therefore was not assessed (Figure 4.3A). Tenascin-C was not assessed as it was not statistically significant between responders and non-responders (Figure 4.3F). Individual ROC curves are shown in Figure 4.8, and output from the ROC curve analysis is shown in Table 4.1. Youden's index was used to assess the overall discriminative power of each measurement, and to assign a cut-off value with maximum sensitivity and specificity. Diagnostic accuracy was determined using the threshold cut-off values for AUC described in Methods.

Each of the measurements had a degree of diagnostic accuracy for identifying CPM responders compared to non-responders, with most measurements having good to very good ability. The best overall discrimination was seen using T_{reg} number, which was able to distinguish between responders and non-responders with a sensitivity of 75% and a specificity of 84.21%.

Measurement	AUC (95%CI, p-value)	Sensitivity (%)	Specificity (%)	Cut-Off Value	Diagnostic Accuracy (AUC) CPM Response
$CD3^+CD4^+$ cells (/µl blood)	0.76 (0.58-0.94, p=0.034)	100	57.89	654.5 (cells/µl blood)	**good**
MMP-3 (pg/ml)	0.80 (0.63-0.96, p=0.017)	100	57.89	22,192 (pg/ml)	**very good**
TIMP-1 (pg/ml)	0.69 (0.49-0.89, p=0.124)	100	63.16	325,772 (pg/ml)	**sufficient**
T_{reg} cells (/µl blood)	0.84 (0.68-0.99, p=0.006)	75	84.21	45.5 (cells/µl blood)	**very good**
WBC ($x10^9$/L)	0.74 (0.55-0.93, p=0.049)	100	63.16	5.95 ($x10^9$/L)	**good**

Table 4.1 ROC curve analysis for immunological and plasma protein measurements significantly altered between CPM responders (n=8) and non-responders (n=19) from TaCTiCC.

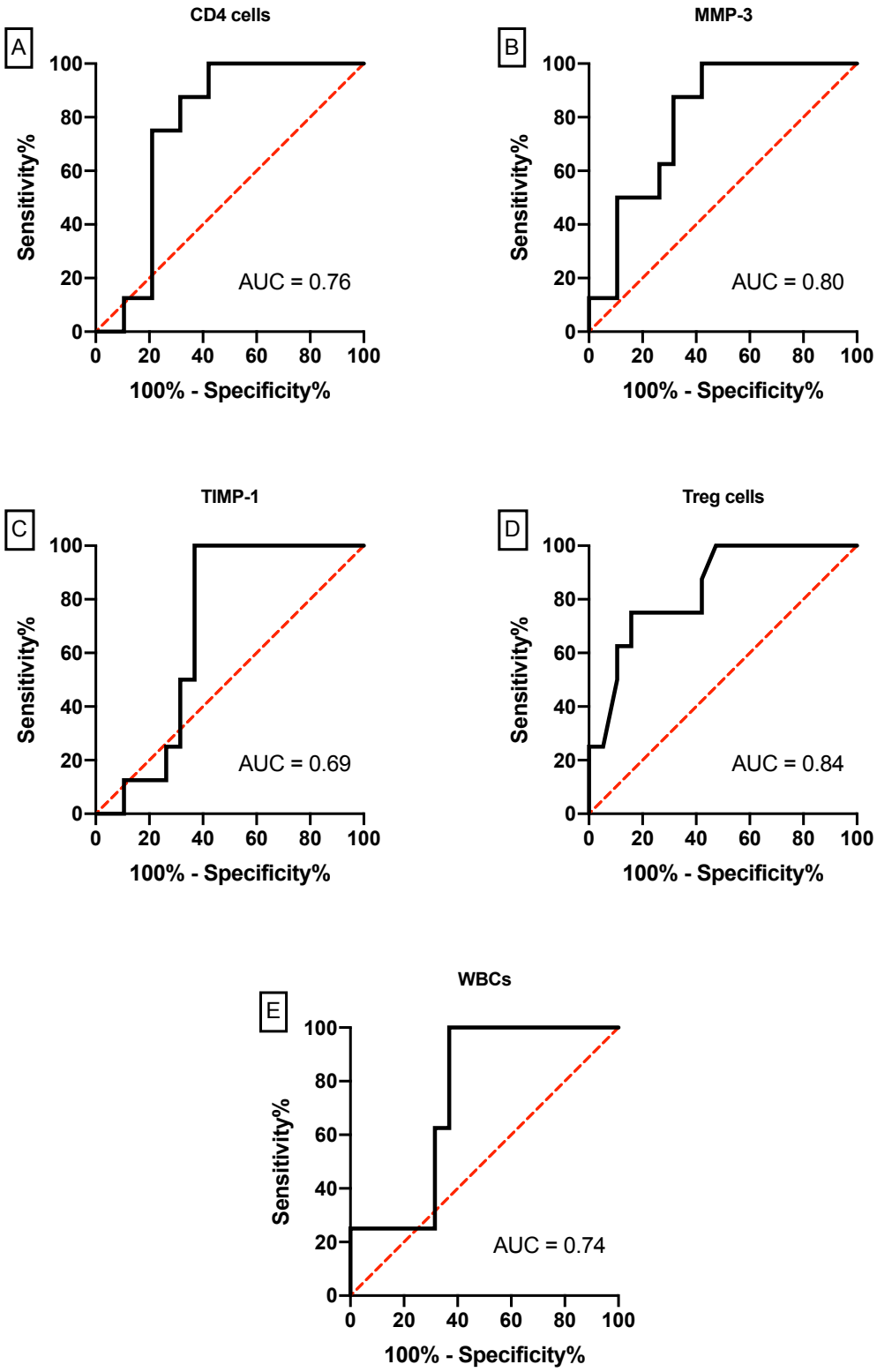


Figure 4.8 (A-E) Immune cell numbers and plasma matrix protein levels have the individual capacity to predict positive response to CPM immunotherapy in mCRC TaCTiCC patients.

ROC curves were generated for CD4s, T_{regs}, WBCs, and plasma MMP-3 and TIMP-1 using measured levels in CPM responders (n=8) and CPM non-responders (n=19) from TaCTiCC. Area under curve as shown.

4.2.4 Modelling Response to Cyclophosphamide Using Multiple Pre-Treatment Immunological and Plasma Protein Measurements

Section 4.2.1 identified several immunological and plasma protein measurements which are significantly altered between TaCTiCC CPM responders and non-responders. Moreover, Section 4.2.3 demonstrated that several of these measurements have a degree of diagnostic sensitivity and specificity for identifying CPM responders. It was hypothesised that a combination of markers would improve prediction sensitivity and/or specificity. This was investigated by logistic regression in R, with CPM response as the binary outcome variable. The following measurements were used to create the model:

1. $CD3^+CD4^+$cell number
2. T_{reg} cell number
3. WBC number
4. Plasma MMP-3

The above measurements were both significantly altered between CPM responders and non-responders in Section 4.2.1 and showed significant diagnostic ability to differentiate between responders and non-responders in Section 4.2.3.

Multicollinearity can cause problems with regression analysis, therefore, to assess the levels of correlation between variables, Spearman's rank order correlation was performed. The resulting correlation matrix is shown in Figure 4.9. There was a moderate positive correlation between WBC count and T_{reg} number, and WBC count and $CD3^+CD4^+$ number. In addition, there was a high positive correlation between T_{reg} and $CD3^+CD4^+$ cell number. Logistic regression models were therefore built using the following combinations of measurements, in order to eliminate multicollinearity within the model:

1. MMP-3 and $CD3^+CD4^+$ cell number
2. MMP-3 and T_{reg} number
3. MMP-3 and WBC number

Of these models, only two resulted in both factors close to statistical significance within the model: MMP-3 and $CD3^+CD4^+$ cells, and MMP-3 and WBCs. Output for these models is shown in Tables 4.2-4.4. Using the derived logistic predictor scores, ROC curve analysis was performed for both models (Figure 4.10). The overall diagnostic

capacity of these models was improved than for each factor individually (Figure 4.8 versus Figure 4.10).

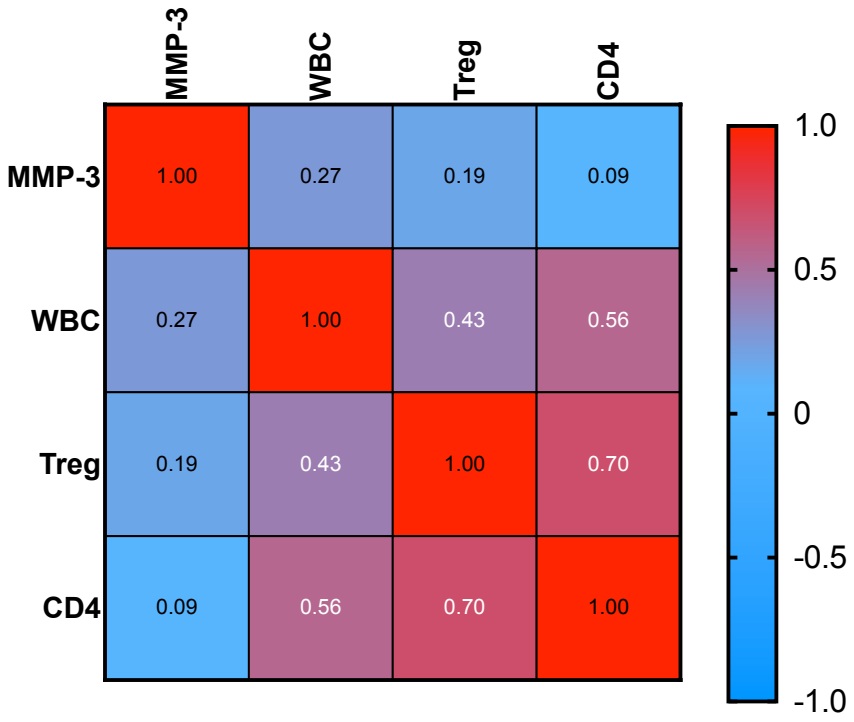


Figure 4.9 Correlation matrix of significantly altered measurements between CPM responders (n=8) and non-responders (n=19).

Nonparametric Spearman's rank order correlation was performed between $CD3^+CD4^+$ cells, T_{reg} cells, WBCs, and plasma MMP-3 level. Spearman rs as shown and interpreted as per methods.

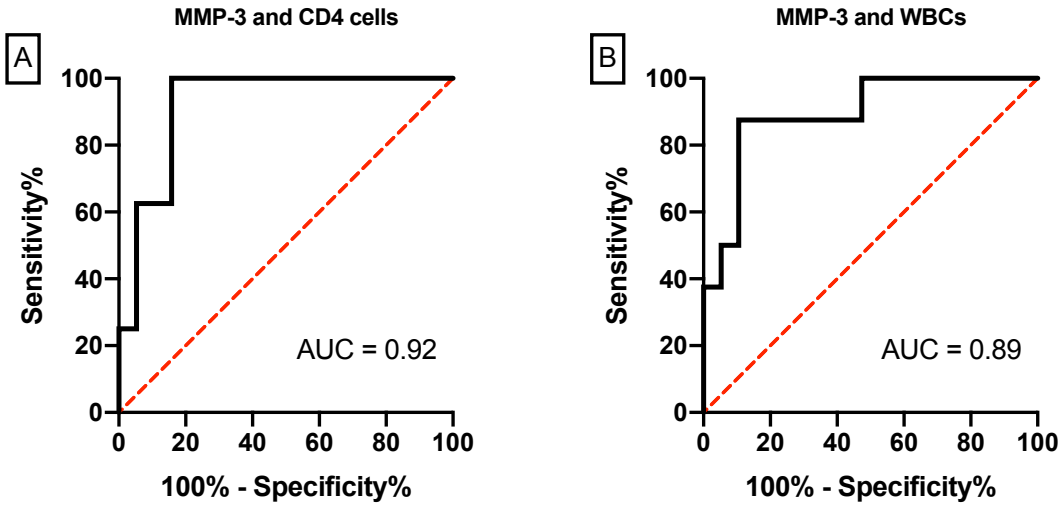


Figure 4.10 (A-B) Combination of $CD3^+CD4^+$ or WBC number with plasma matrix protein MMP-3 concentration has high diagnostic capacity for predicting response to CPM immunotherapy in mCRC TaCTiCC patients. ROC curves were plotted using logistic predictor scores generated from each model for CPM responders (n=8) and non-responders (n=19). Area under curve as shown.

Measurement	AUC (95%CI, p-value)	Sensitivity (%)	Specificity (%)	Cut-Off Logistic Predictor	Diagnostic Accuracy (AUC) CPM Response
MMP-3 and $CD3^+CD4^+$	0.92 (0.82-1, p=0.0007)	100	84.21	0.74	**excellent**
MMP-3 and WBCs	0.89 (0.76-1, p=0.0014)	87.5	89.47	0.67	**very good**

Table 4.2 ROC curve analysis for logistic regression models to identify TaCTiCC CPM responders (n=8) compared to non-responders (n=19).

Model Variables	Estimate	p value
MMP-3 (ng/ml)	0.171	0.055
$CD3^+CD4^+$ cells (/ul blood)	0.007	0.033 (*)

Table 4.3 Output of logistic regression model for mCRC response to CPM treatment using MMP-3 and $CD3^+CD4^+$ cell number as variables.

A logistic regression was performed using response to CPM as a binary variable (responders n=8 and non-responders n=19). Estimate and p value as shown.

Model Variables	Estimate	p value
MMP-3 (ng/ml)	0.159	0.052
WBC ($x10^9$/L)	0.837	0.068

Table 4.4 Output of logistic regression model for mCRC response to CPM treatment using MMP-3 and WBC number as variables.

A logistic regression was performed using response to CPM as a binary variable (responders n=8 and non-responders n=19). Estimate and p value as shown.

4.2.5 Changes in Pre-treatment Cyclophosphamide Response Biomarkers in Advanced Metastatic CRC Patients During Treatment

In order to explore whether treatment affects the levels of the identified markers of CPM response, and whether the differences between responders/non-responders are maintained through treatment, the markers were next assessed across the course of treatment. This analysis was performed from pre-treatment (TD1) until day 22 (TD22), in the same patient cohort. Basophils and WBCs were only measured pre-treatment, and Tenascin-C did not each statistical significance between responders and non-responders, therefore only the following were included in this analysis:

1. $CD3^+CD4^+$ cell number
2. T_{reg} cell number
3. Plasma MMP-3
4. Plasma TIMP-1

Pre-treatment MMP-3 was significantly higher in non-responders and this difference was maintained at TD8, TD15, and TD18 (Figure 4.7A, significance not shown). There was, however, no change in MMP-3 levels in either responders or non-responders from TD1 to TD22, at each of the assessed time points (Figure 4.11A). Similarly, pre-treatment and TD8 TIMP-1 was significantly higher in non-responders (Figure 4.11B, significance not shown), and there was no change in TIMP-1 level in either responders or non-responders across CPM treatment (Figure 4.11B). There was no significant change in the level of either MMP-3 or TIMP-1 in non-treated controls, between TD1 and TD22 (data not shown).

When considering the immunological measurements, $CD3^+CD4^+$ cell number was significantly higher pre-treatment and at TD18 in non-responders (Figure 4.11C, significance not shown). In non-responders there was also a significant decrease in $CD3^+CD4^+$ number between TD1 and TD22 (Figure 4.11C). Non-responders had significantly higher T_{reg} cells pre-treatment, and at TD18 and TD22 (Figure 4.11D, significance not shown). There was also a significant decrease in T_{reg} number in non-responders between TD1 and TD15, TD18 and TD22, and in responders between TD1 and TD18 (Figure 4.11D). No significant difference was found in $CD3^+CD4^+$ or T_{reg} cell number in non-treated controls between TD1 and TD22 (data not shown).

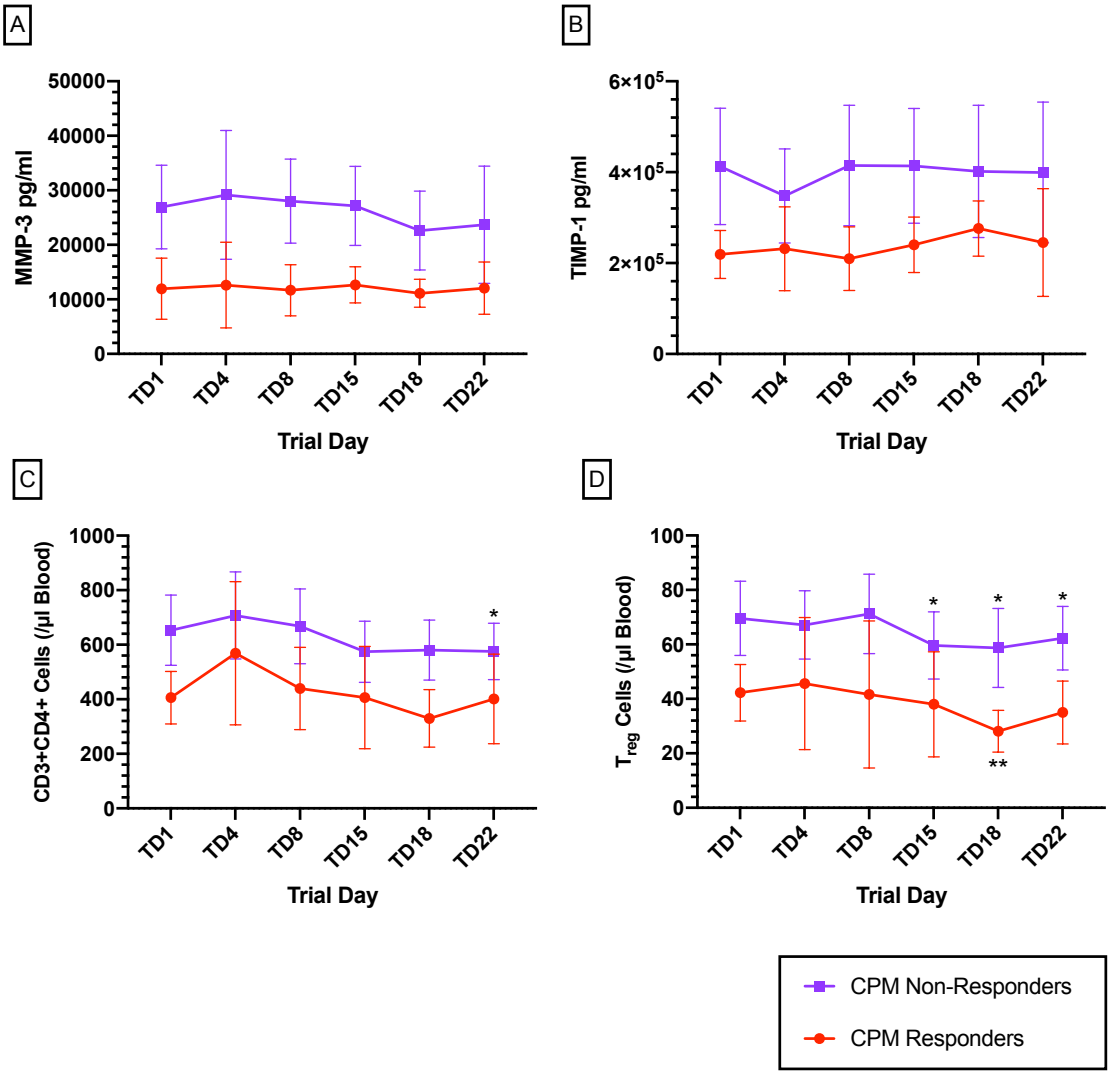


Figure 4.11 (A-D) Changes in plasma MMP-3 and TIMP-1, and $CD3^+CD4^+$ and T_{reg} cell number in mCRC patients across CPM-treatment from TD1 to TD22. TaCTiCC patients receiving CPM-treatment between TD1 and TD22 were split into those who responded to therapy (n=5-8) and those who did not respond to therapy (n=17-19) based on both magnitude of T_{reg} depletion and IFNγ response to 5T4. For each group, differences between TD1 and each subsequent trial day were compared using Wilcoxon signed-rank test. Mean with 95% confidence interval shown. Asterisk indications of significance are as per methods and indicate significant differences of the respective time point compared to TD1 of that group.

4.2.6 Association of Pre-Treatment Cyclophosphamide Response Biomarkers with Patient Clinical Parameters

The results of this study thus far have identified $CD3^+CD4^+$ and T_{reg} cell numbers, and plasma MMP-3 and TIMP-1 as candidate biomarkers of response to CPM treatment. In order to investigate the physiological relevance of these in advanced mCRC, their association with the following patient clinical parameters was explored:

1. Age
2. Sex
3. Primary Tumour Location
4. Site of Metastasis

Correlations between age and each response marker were assessed using Spearman's rank correlation coefficient in the entire TaCTiCC cohort (n=52). None of the markers significantly correlated with age. When the cohort was split based on sex, there was no significant difference in the levels of the markers between males (n=38) or females (n=14).

When the 52 TaCTiCC patients were split based on primary tumour location (left-handed, right-handed, or rectal), $CD3^+CD4^+$ and T_{reg} cell numbers were significantly higher in patients with right-handed tumours than left-handed or rectal tumours (Figure 4.12 A&C). When considering only the CPM-treated patients from Groups 2 and 4, there were significantly higher $CD3^+CD4^+$ cell numbers in patients with right-handed tumours than those with rectal tumours (Figure 4.12 B).

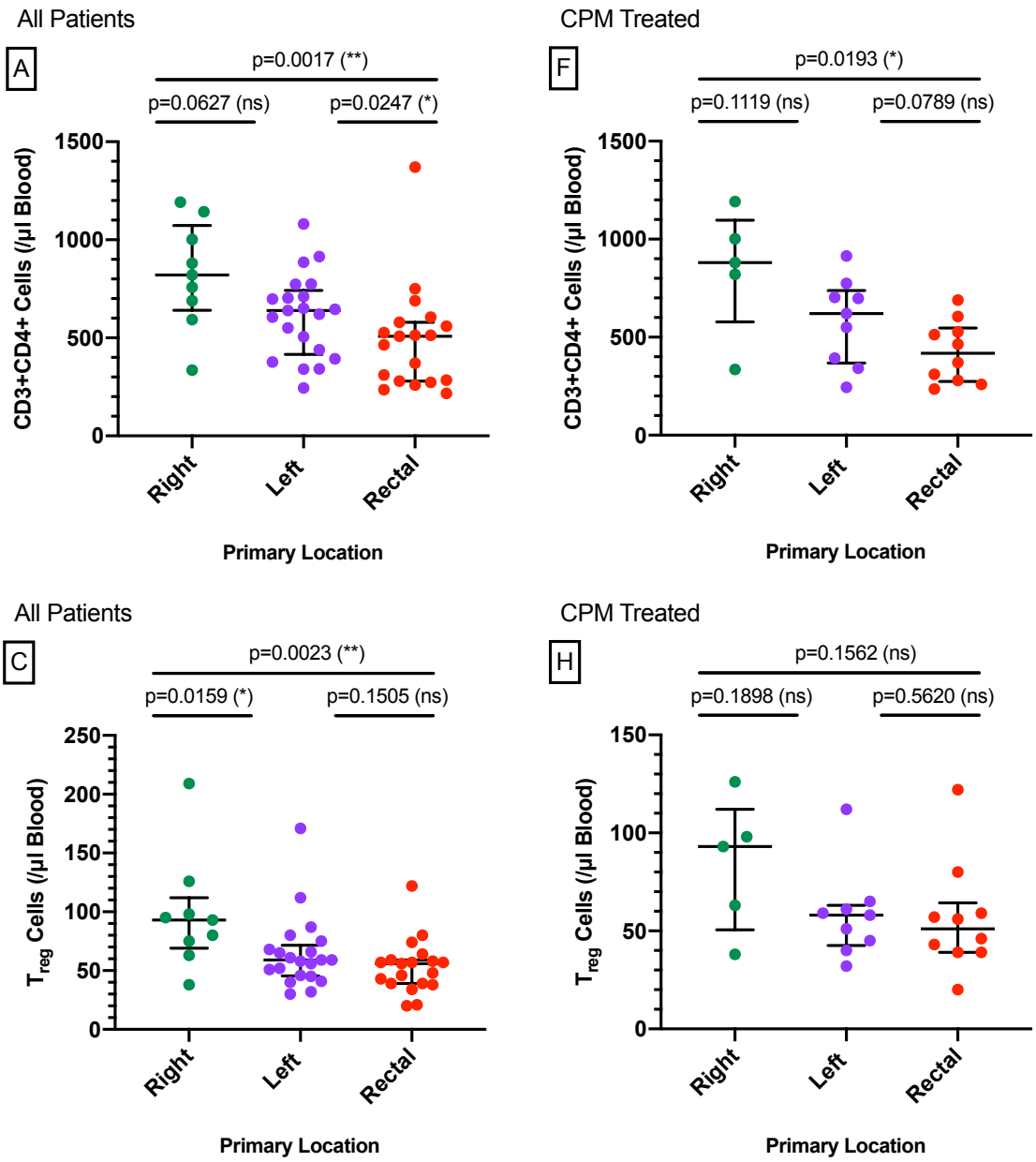


Figure 4.12 (A-D) $CD3^+CD4^+$ and T_{reg} number are significantly higher in advanced mCRC patients with right-handed primary tumours.

Using the TaCTiCC cohort, patients were split based on primary tumour location. $CD3^+CD4^+$ and T_{reg} cell numbers were compared in patients from all trial groups (A and C, n=49), and those receiving CPM-treatment (B and D, n=24). Median and interquartile range shown.

To investigate the association of the identified markers with site of metastasis, patients were stratified by presence/absence of lesions in liver, peritoneum, or lung, considering each location individually. None of the markers were significantly different between patients with and without lung metastasis. MMP-3 and TIMP-1, however, were significantly higher in patients with liver metastasis (Figure 4.13 A-D). When CPM- patients were split into responders (n=8) and non-responders (n=19), and patients with liver metastasis omitted from the analysis, there was no significant difference between the remaining responders/non-responders (Figure 4.14). These remaining patients also had generally lower levels of TIMP-1 than the others. $CD3^+CD4^+$ and T_{reg} cell numbers were significantly lower in patients with peritoneal metastasis (Figure 4.15 A-D).

The results of Figures 4.13 to 4.15 are confounded by the presence of multiple metastatic lesions within many patients. To address this, only TaCTiCC patients with one distinguishable metastatic lesion were next considered and separated based on tumour location. MMP-3 and TIMP-1 levels remained higher in patients with liver metastasis, and $CD3^+CD4^+$ and T_{reg} cell numbers were generally lower in patients with peritoneal metastasis than liver metastasis (Figure 4.16 A-H).

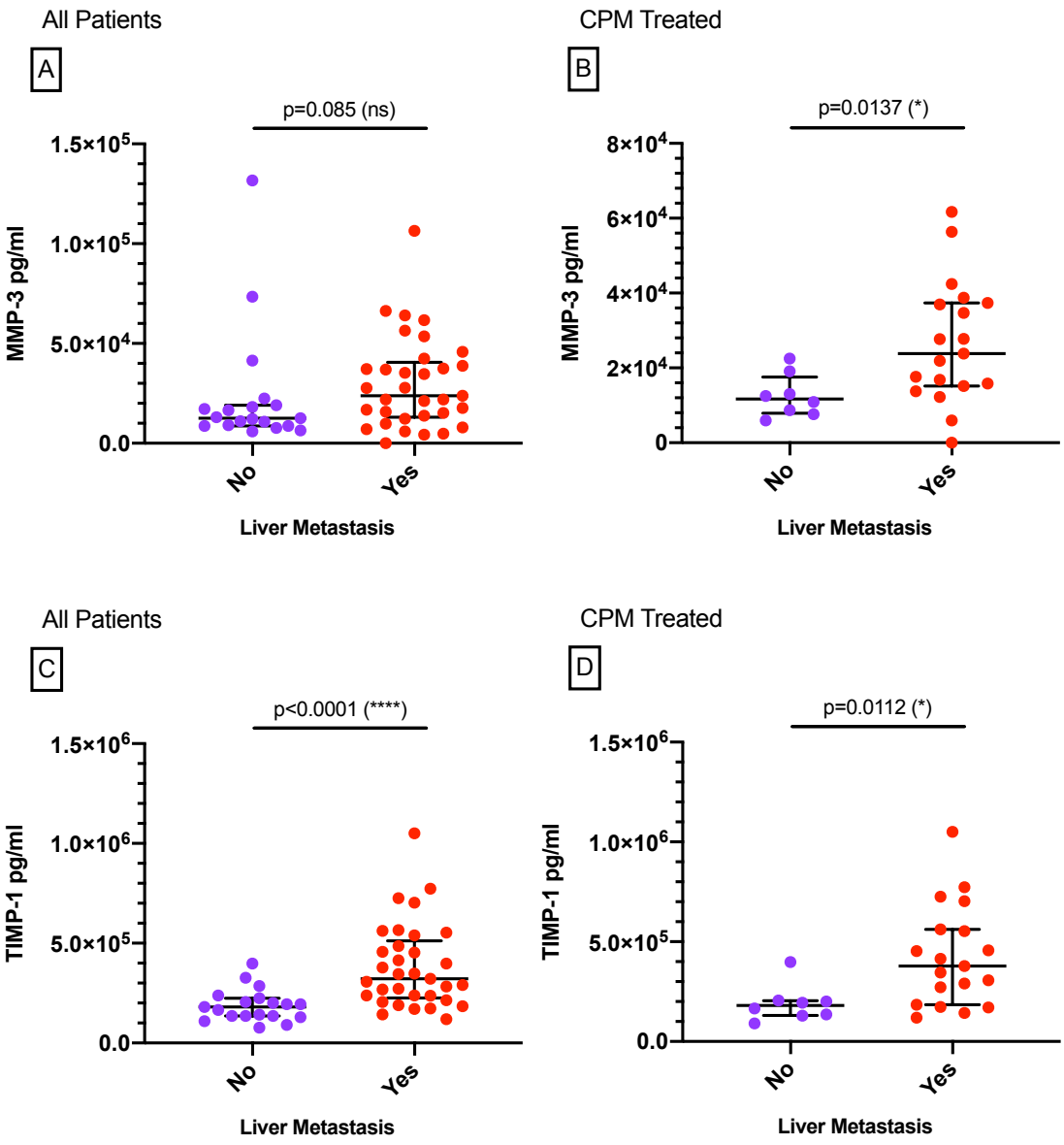


Figure 4.13 (A-D) Plasma MMP-3 and TIMP-1 are significantly higher in advanced mCRC patients with measurable liver metastasis.

Using the TaCTiCC cohort, patients were split into those with measurable liver metastasis prior to treatment, and those without. The levels of MMP-3 and TIMP-1 were compared between all patients (A and C, n=52, TaCTiCC Groups 1-4), and those receiving CPM treatment (B and D, n=27, TaCTiCC Groups 2 and 4). Median and interquartile range shown.

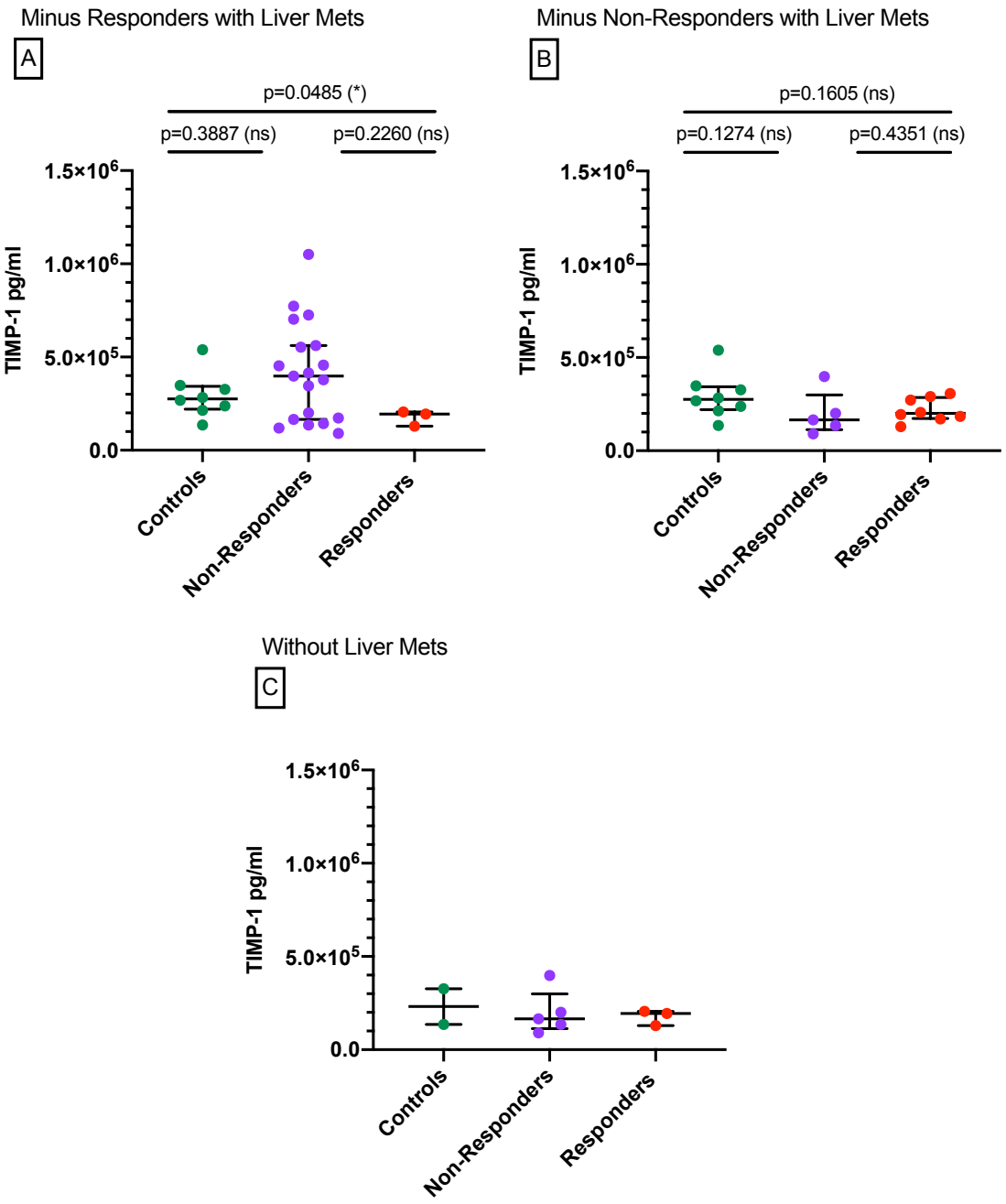


Figure 4.14 (A-D) Increased plasma TIMP-1 is associated with liver metastasis in mCRC patients, regardless of response to CPM treatment. CPM-treated patients were split into those who responded to therapy (n=8), those who did not (n=19), or untreated controls (n=8) based on magnitude of T_{reg} depletion and IFNγ response to 5T4. Either responders (A), non-responders (B) or all patients (C) with detectable liver metastasis were omitted from the analysis to assess differences between the groups. Median and interquartile range shown.

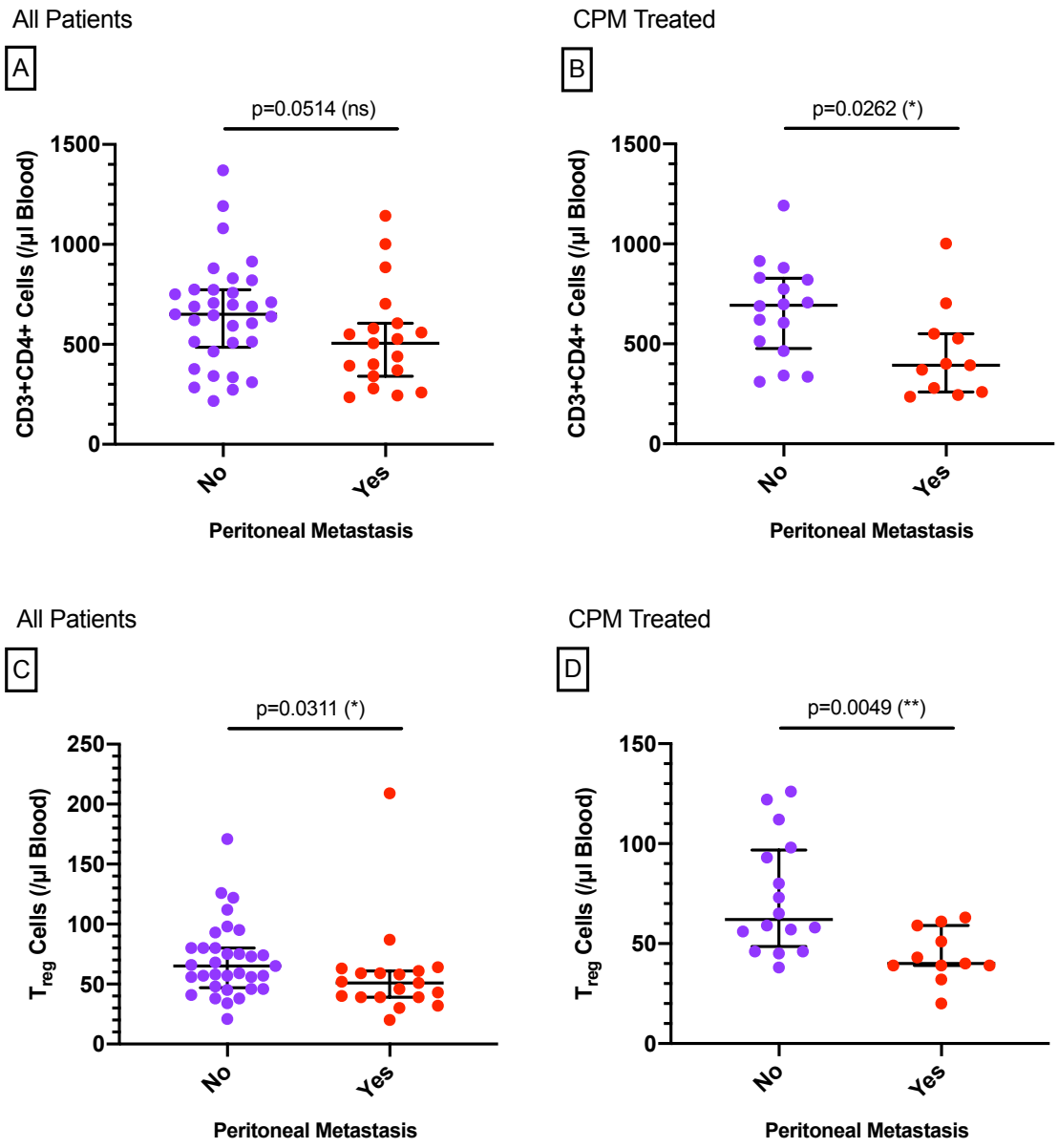
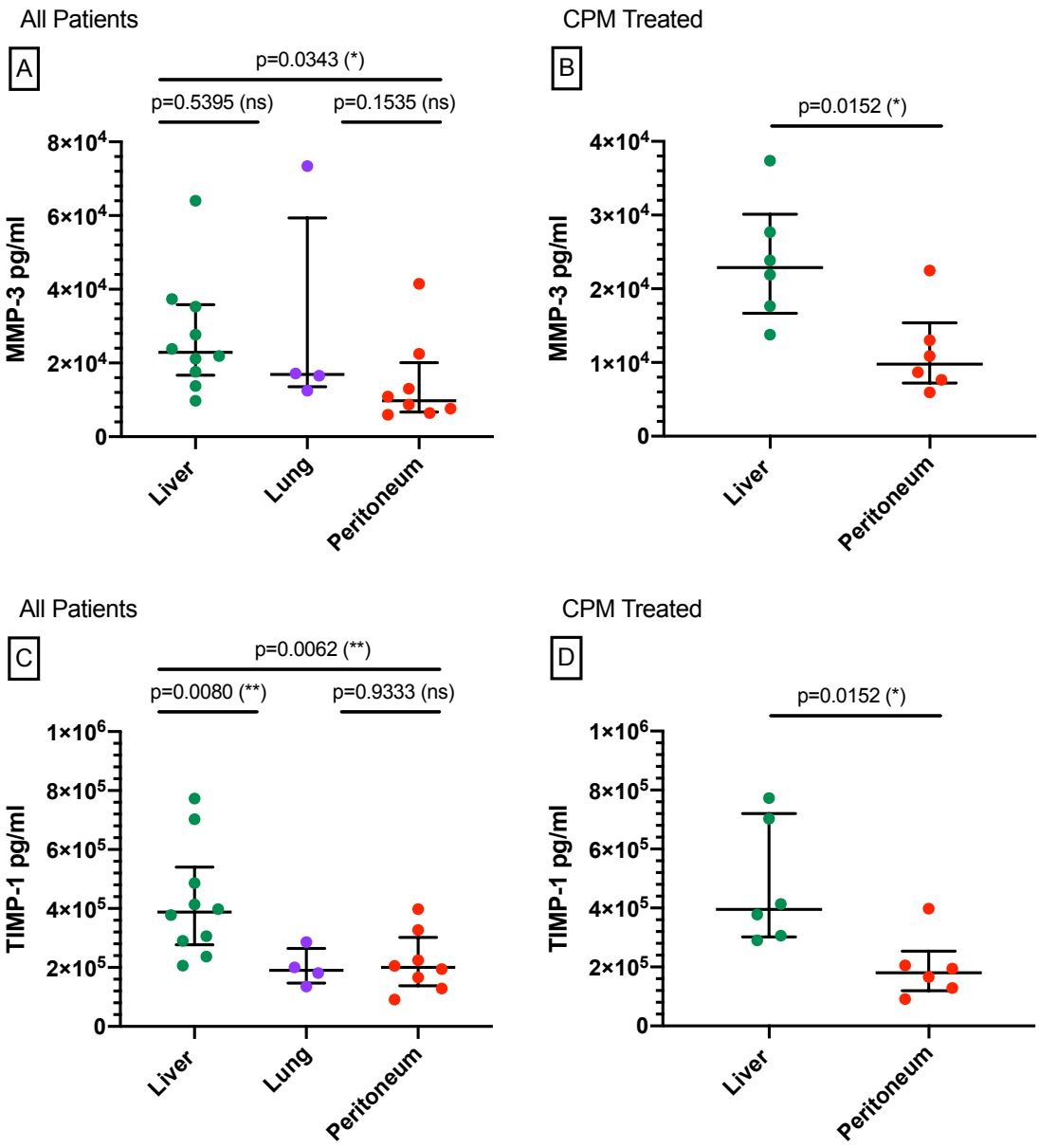


Figure 4.15 (A-D) $CD3^+CD4^+$ and T_{reg} number are significantly lower in advanced mCRC patients with measurable peritoneal metastasis.

Using the TaCTiCC cohort, patients were split into those with measurable peritoneal metastasis prior to treatment, and those without. The numbers of $CD3^+CD4^+$ and T_{reg} cells were compared between all patients (A and C, n=52, TaCTiCC Groups 1-4), and those receiving CPM treatment (B and D, n=27, TaCTiCC Groups 2 and 4). Median and interquartile range shown.


All Patients
A
p=0.0343 (*)
p=0.5395 (ns)
p=0.1535 (ns)
MMP-3 pg/ml
8×10^4
6×10^4
4×10^4
2×10^4
0
Liver
Lung
Peritoneum
CPM Treated
B
p=0.0152 (*)
MMP-3 pg/ml
4×10^4
3×10^4
2×10^4
1×10^4
0
Liver
Peritoneum
All Patients
C
p=0.0062 (**)
p=0.0080 (**)
p=0.9333 (ns)
TIMP-1 pg/ml
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
Liver
Lung
Peritoneum
CPM Treated
D
p=0.0152 (*)
TIMP-1 pg/ml
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
Liver
Peritoneum

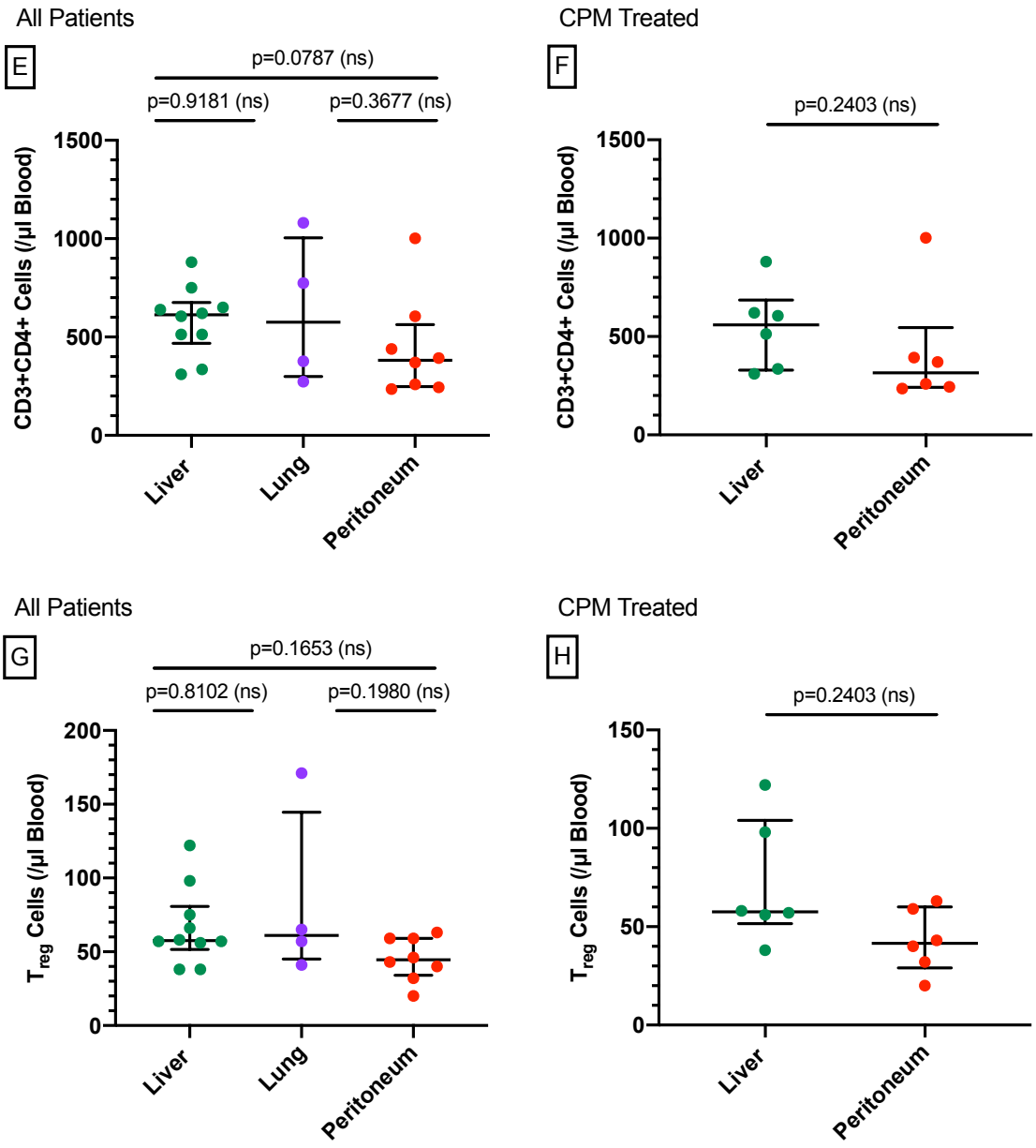


Figure 4.16 (A-H) Advanced mCRC patients have differences in plasma MMP-3 and TIMP-1, and $CD3^+CD4^+$ and T_{reg} cell number based on first site of metastasis.

Using TaCTiCC patients with only one metastatic site, patients were split based on its location. The levels of plasma MMP-3 and TIMP-1, and $CD3^+CD4^+$ and T_{reg} cell numbers were compared in these patients from all trial groups (A, C, E and G, n=23), and those receiving CPM-treatment (B, D, F, and H, n=12). Median and interquartile range shown.

4.2.7 Comparison of Pre-Treatment Serological, Immunological and Plasma Protein Measurements Between TroVax Responders and Non-Responders

To assess the usefulness of pre-treatment serological, immunological and plasma protein markers for predicting response to TroVax, levels between TaCTiCC responders and non-responders were compared. These parameters were the same as for Section 4.2.1. This analysis was performed using pre-trial measurements, and trial day 22 measurements, immediately before the first TroVax injection. As CPM treatment was given to a subset of patients prior to TroVax, patients receiving TroVax monotherapy and those receiving combined TroVax/CPM therapy were considered both together and independently. In the TaCTiCC clinical trial, response to TroVax was defined as patients who, at any treatment day, both:

1. Doubled cultured T cell response to 5T4.
2. Doubled antibody response to 5T4.

When considering all TaCTiCC patients receiving TroVax as part of their treatment, the pre-treatment (TD1) levels of CCL17 were significantly decreased, and the levels of MMP-7 significantly increased, in patients who did not respond to TroVax (Figure 4.17 A and B). Responders to TroVax treatment had increased IFNγ 5T4 cultured T cell response across treatment. Pre-treatment, however, there was a significantly higher pre-existing cultured IFNγ 5T4 T cell response in non-responder patients (Figure 4.17 C). Immediately prior to the first TroVax injection (TD22), CCL17 and MMP-7 were not significantly different between responders and non-responders (data not shown), however, the plasma level of MMP-8 was significantly increased in non-responders (Figure 4.17 D).

As the results in Figure 4.17 are confounded by the fact that a subset of the patients received CPM treatment prior to TroVax, individuals receiving only TroVax monotherapy were next considered alone. TD1 plasma levels of CCL17 were significantly decreased, and the levels of MMP-7 significantly increased, in patients who did not respond to TroVax (Figure 4.18 A and B). When considering TD22, immediately before TroVax, there was a trend towards higher levels of CCL17 in patients who responded to therapy (Figure 4.18 C).

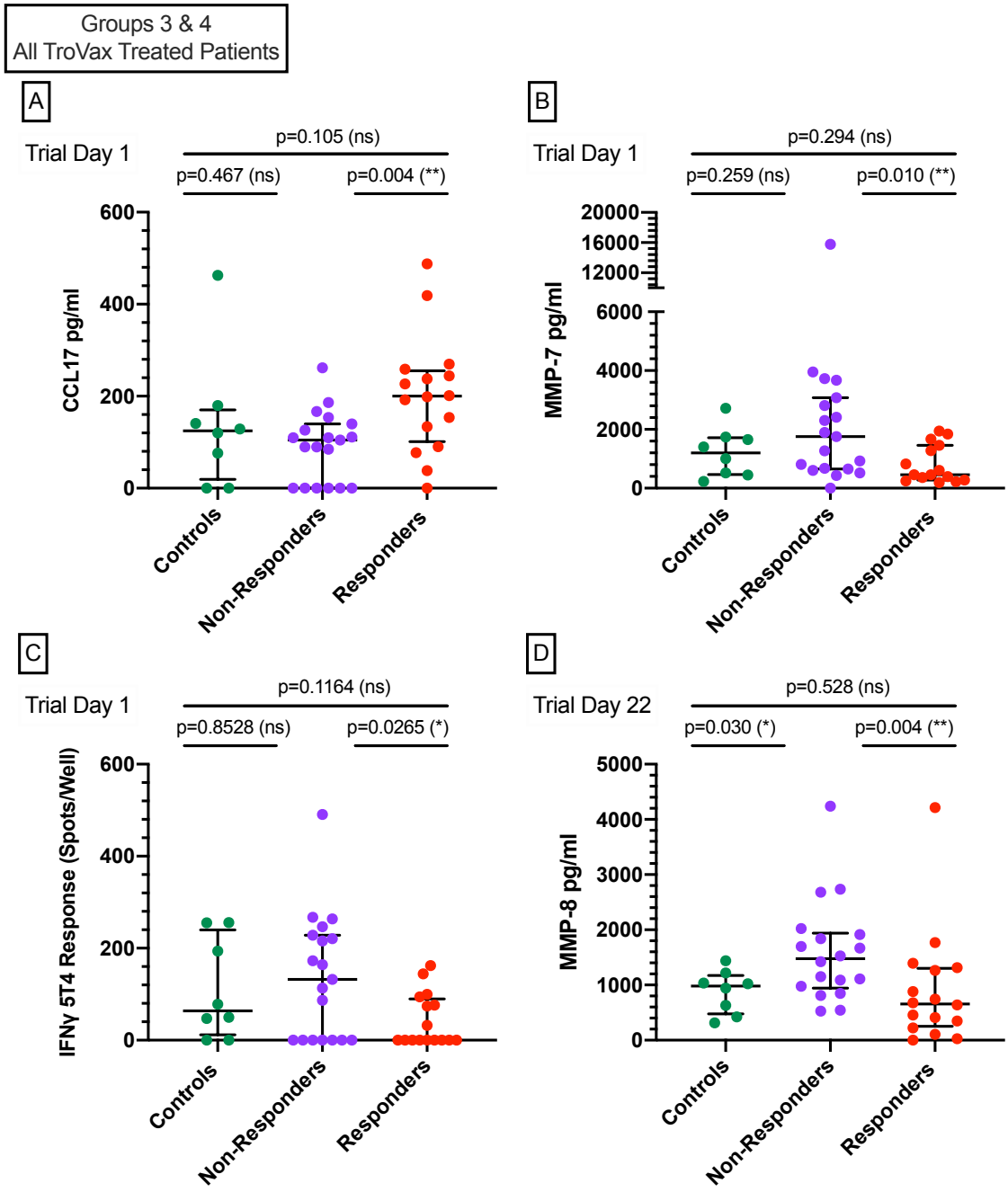


Figure 4.17 (A-D) TD1 plasma levels of CCL17 and MMP-7, magnitude of IFNγ T cell response to 5T4, and TD22 MMP-8 are significantly different between mCRC patients who respond/do not respond to TroVax immunotherapy.

TroVax treated patients from combined TaCTiCC Group 3 (receiving TroVax monotherapy), and Group 4 (receiving dual CPM and TroVax therapy) were split into those who responded to therapy (n=16), those who did not (n=19), based on 5T4 antibody response, and magnitude of cultured IFNγ T cell response to 5T4. Untreated control patients are also shown (n=8). Median and interquartile range shown.

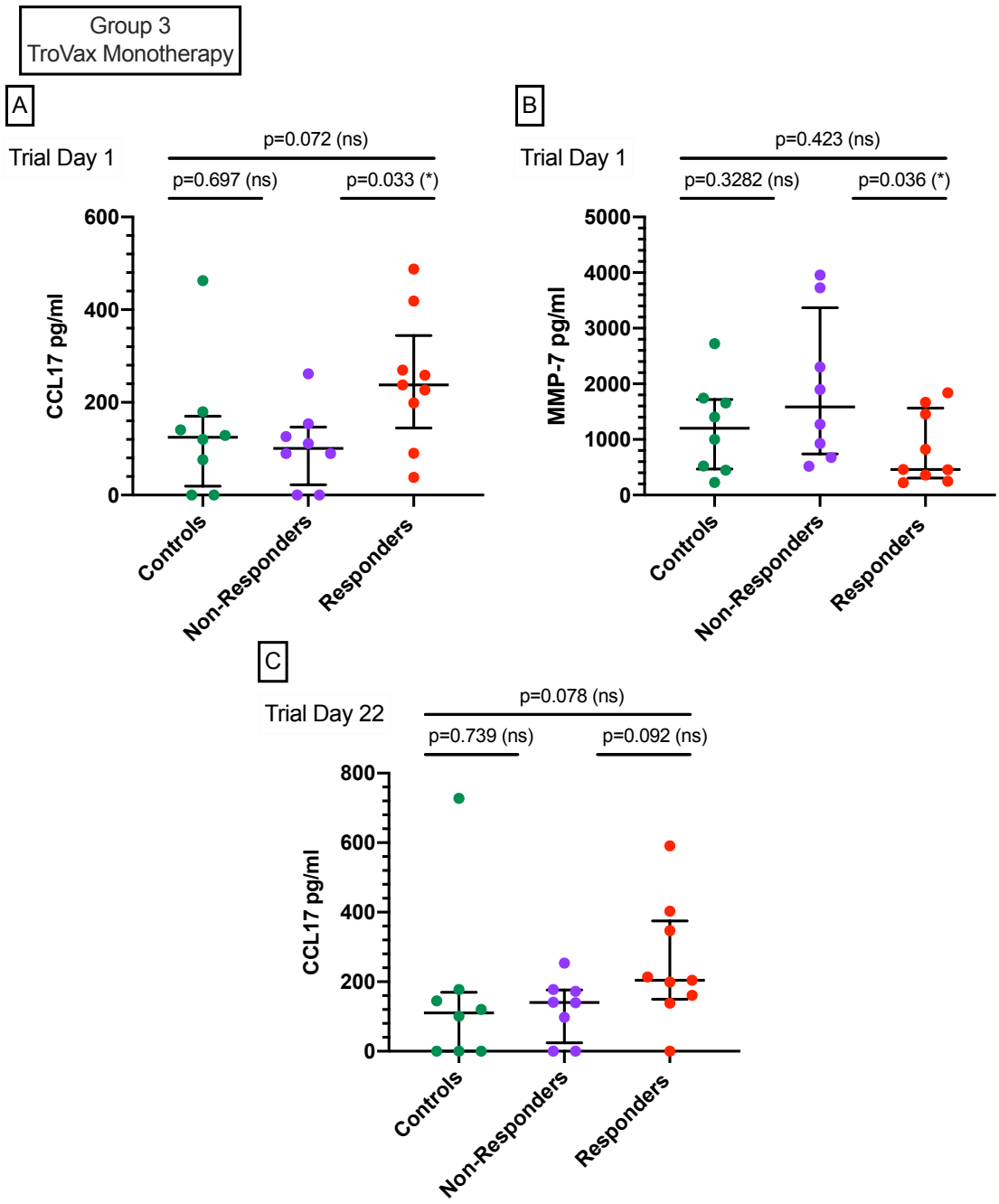
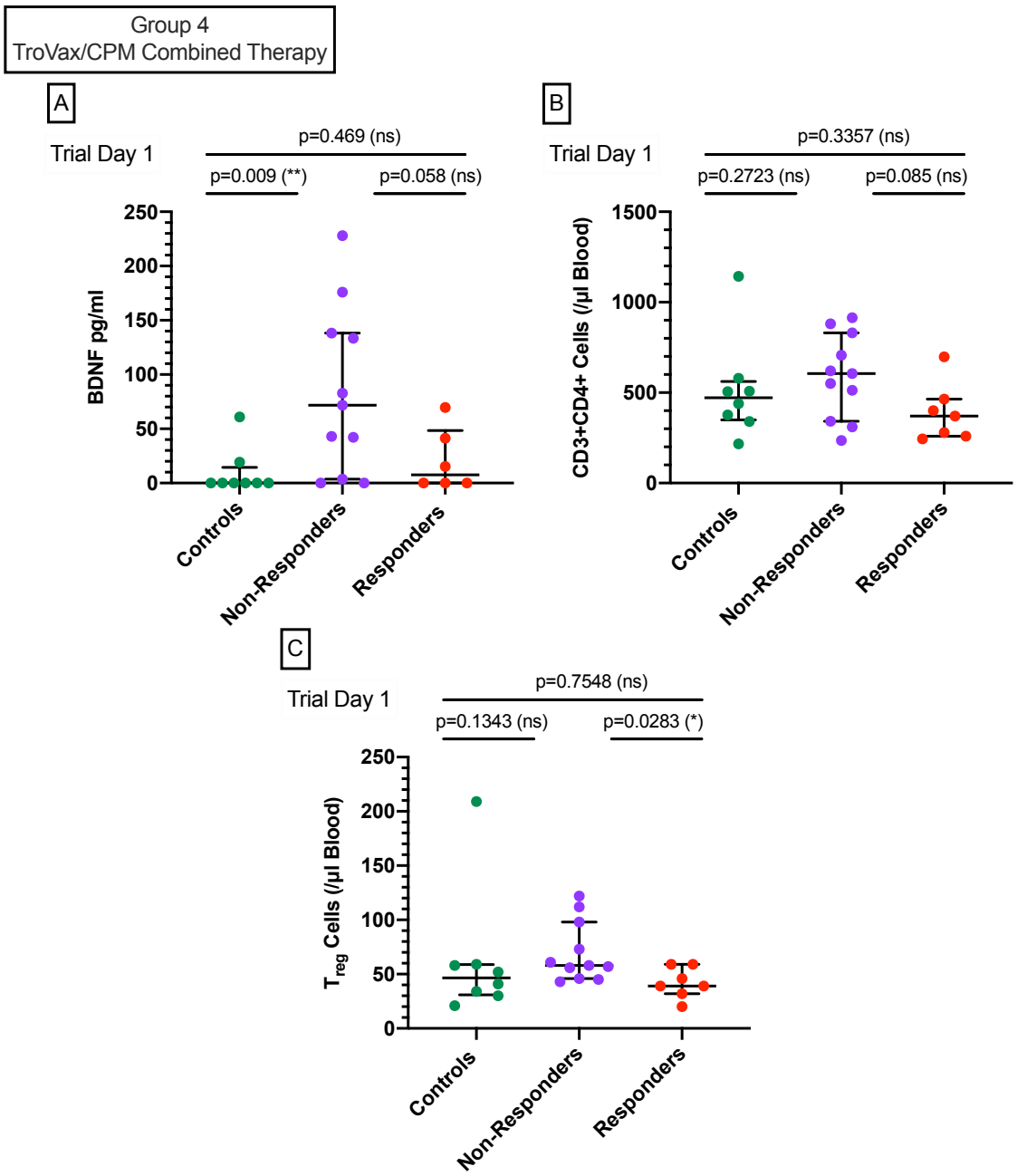


Figure 4.18 (A-C) TD1 plasma levels of CCL17 and MMP-7, and TD22 plasma levels of CCL17 are different, between mCRC patients who respond/do not respond to TroVax immunotherapy.

TroVax treated patients from TaCTiCC Group 3 (receiving TroVax monotherapy) were split into those who responded to therapy (n=9), those who did not (n=8), based on 5T4 antibody response, and magnitude of cultured IFNγ T cell response to 5T4. Untreated control patients are also shown (n=8). Median and interquartile range shown.

Next, TaCTiCC patients receiving dual CPM and TroVax treatment (Group 4) were considered alone. At TD1, there was an increase in plasma BDNF, and in $CD3^+CD4^+$ and T_{reg} cell number in patients who did not respond to TroVax therapy (Figure 4.19 A-C). At TD22, immediately before TroVax treatment, there was a significant increase in plasma MMP-8 in patients who did not respond to therapy (Figure 4.19 D). Plasma MMP-10 was also increased in these non-responders (Figure 4.19 E). Additionally, there was a significantly higher cultured IFNγ 5T4 T cell response, and a higher number of T_{reg} cells in the non-responders at TD22 (Figure 4.19 F and G).


Group 4
TroVax/CPM Combined Therapy
A
Trial Day 1
p=0.469 (ns)
p=0.009 (**)
p=0.058 (ns)
BDNF pg/ml
250
200
150
100
50
0
Controls
Non-Responders
Responders
B
Trial Day 1
p=0.3357 (ns)
p=0.2723 (ns)
p=0.085 (ns)
CD3+CD4+ Cells (/µl Blood)
1500
1000
500
0
Controls
Non-Responders
Responders
C
Trial Day 1
p=0.7548 (ns)
p=0.1343 (ns)
p=0.0283 (*)
Treg Cells (/µl Blood)
250
200
150
100
50
0
Controls
Non-Responders
Responders

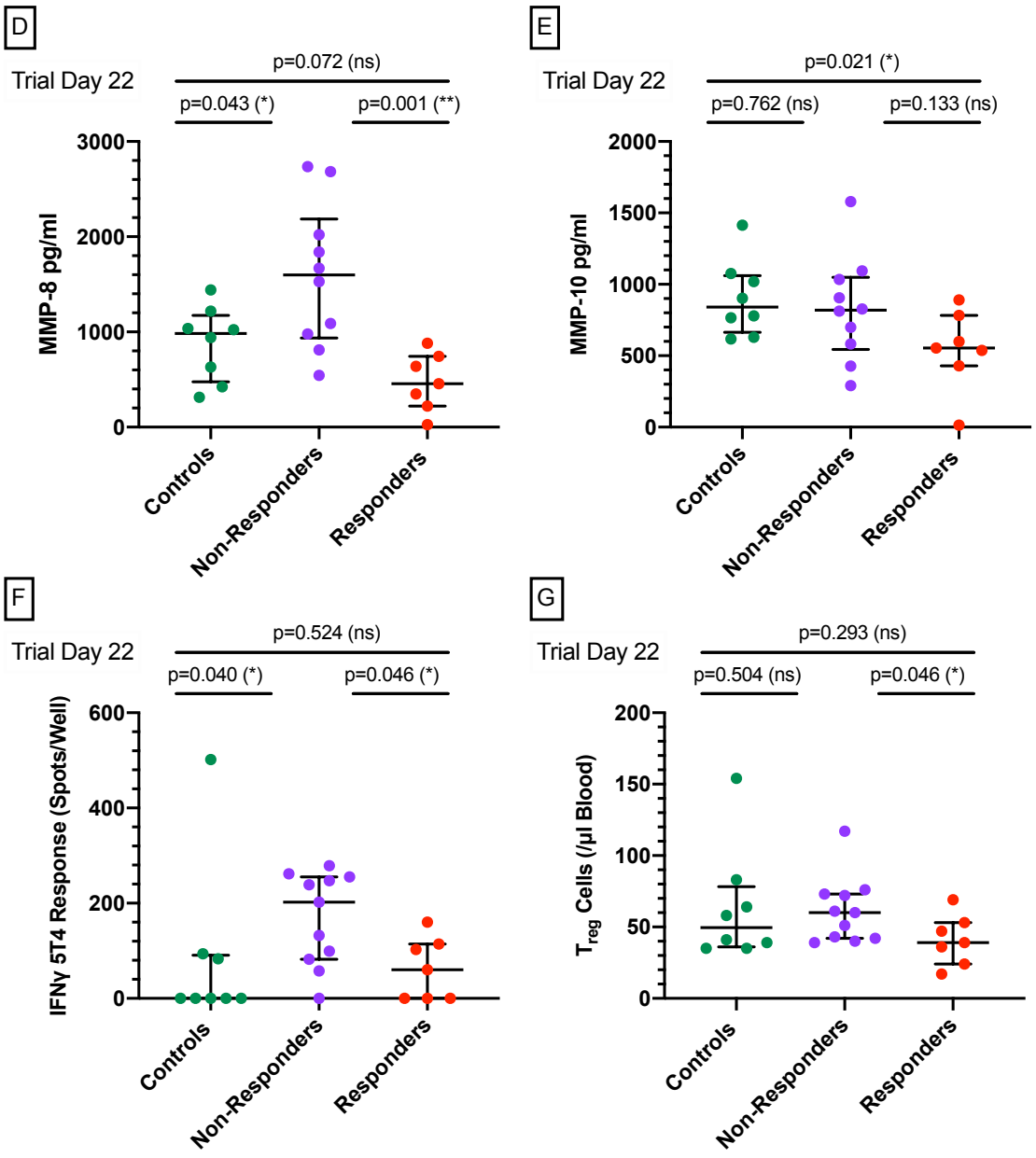


Figure 4.19 (A-G) The levels of several plasma proteins and immune markers are significantly different at TD1 and TD22 in mCRC patients who respond/do not respond to TroVax immunotherapy.

TroVax treated patients from TaCTiCC Group 4 (receiving CPM and TroVax dual therapy) were split into those who responded to therapy (n=6-7), those who did not (n=10-11), based on 5T4 antibody response, and magnitude of cultured IFNγ T cell response to 5T4. Untreated control patients are also shown (n=8). Median and interquartile range shown.

4.2.8 Association of Pre-Treatment Biomarkers of Response to TroVax with Overall and Progression-Free Survival

As previously described, TaCTiCC patients who immunologically responded to therapy had improved survival. It was therefore hypothesised that the identified markers of TroVax response would also predict survival. TroVax treatment was given either alone (Group 3) or subsequent to CPM treatment (Group 4). The markers identified in Section 4.2.7 for predicting TroVax response were different when considering these groups of patients alone, therefore only the markers identified in Group 3 patients were considered in this section; CCL17 and MMP-7.

As CCL17 and MMP-7 are continuous in nature, they were first split into categorical variables to perform this analysis. CCL17 was not significantly altered between healthy donors and mCRC TaCTiCC patients (Chapter 3), therefore it was stratified into high and low based on mean, median, and 75% and 25% quartiles. MMP-7 was stratified into high and low based on the uppermost level measured for healthy individuals as the cut-off, and also by mean, median, 75% and 25% quartiles. Overall and progression-free survival was investigated by low-rank test in CCL17 and MMP-7 high and low groups and displayed by Kaplan-Meier plot.

There was an indication that low CCL17, split above and below the 25% quartile, associates with reduced overall survival when considering the entire TaCTiCC cohort (Figure 4.20). This was not the case when TroVax treated patients were considered alone, or for progression-free survival (data not shown).

High MMP-7, when stratified based on 75% quartile appeared to associate with reduced overall survival in the combined TaCTiCC group (Figure 4.21A). When considering only patients receiving TroVax, there was a significant association with high MMP-7 and reduced overall and progression free survival (Figure 4.21B and 4.22B).

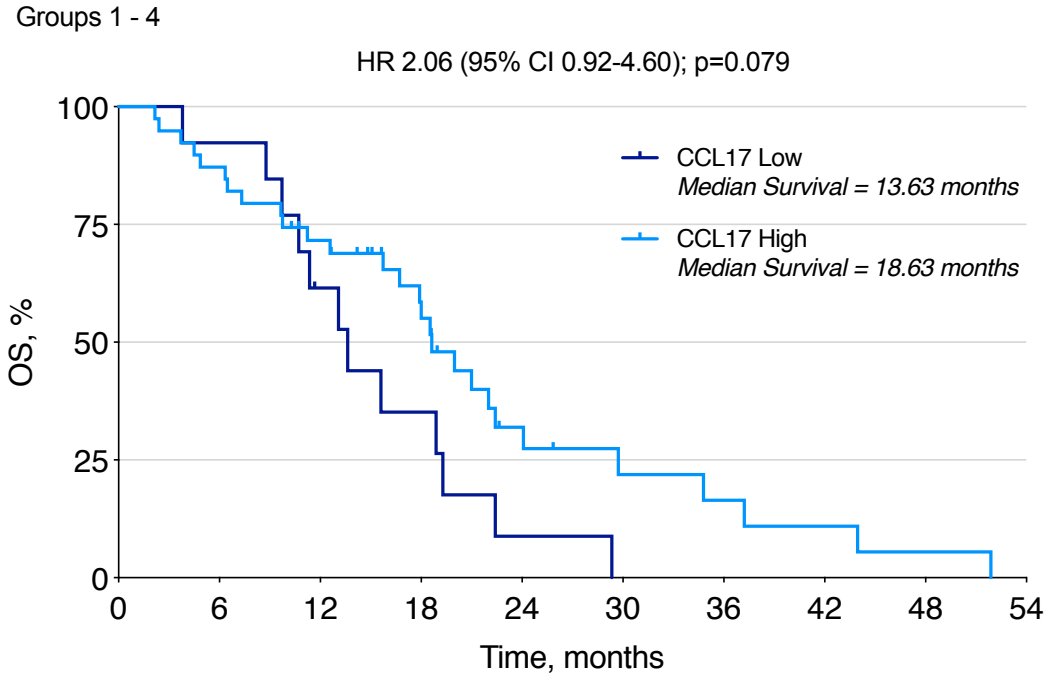


Figure 4.20 Kaplan-Meier survival curves showing overall survival of TaCTiCC patients stratified by high and low CCL17.

mCRC TaCTiCC patients (n=52) were stratified by high and low CCL17 by above and below the 25% quartile. Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown.

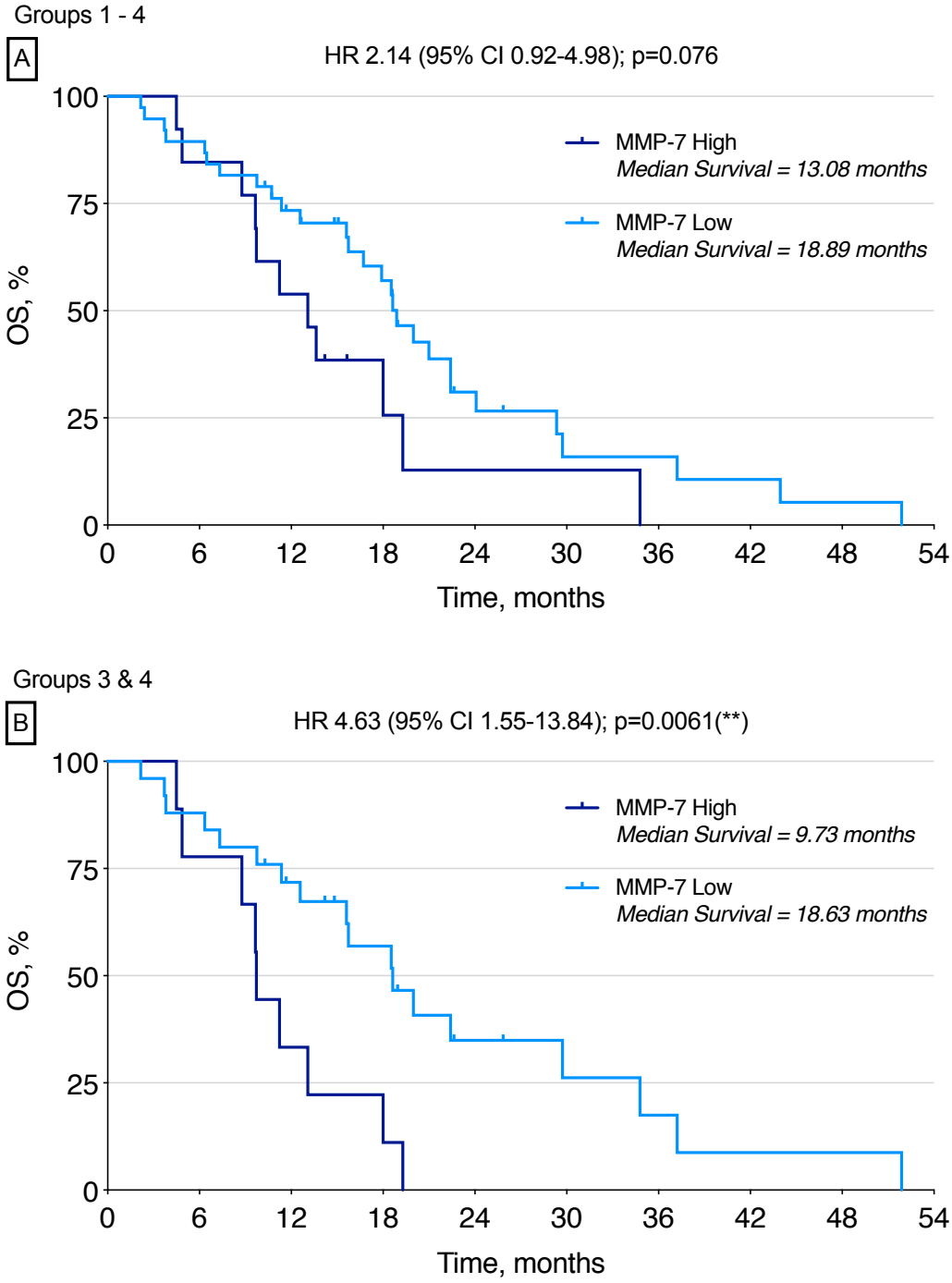


Figure 4.21 (A-B) Kaplan-Meier survival curves showing overall survival of TaCTiCC patients stratified by high and low MMP-7.

mCRC TaCTiCC patients (A, n=51) and TaCTiCC patients receiving TroVax (B, n=34) were stratified by high and low MMP-7 by above and below the 75% quartile. Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown.

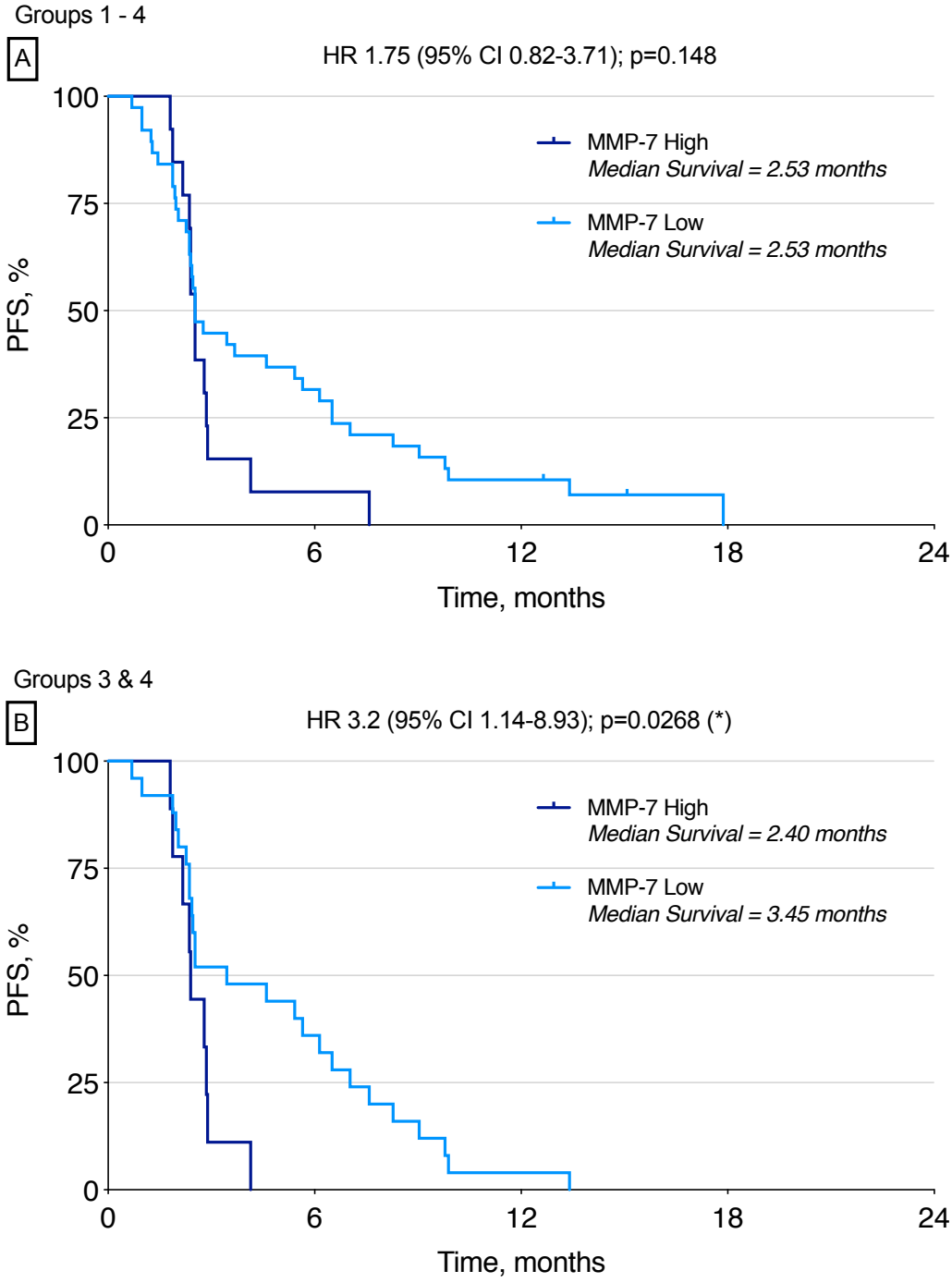


Figure 4.22 (A-B) Kaplan-Meier survival curves showing progression-free survival of TaCTiCC patients stratified by high and low MMP-7.

mCRC TaCTiCC patients (A, n=51) and TaCTiCC patients receiving TroVax (B, n=34) were stratified by high and low MMP-7 by above and below the 75% quartile. Curves were compared by log-rank test, and hazard ratios were computed using the Mantel Haenszel approach. Median survival as shown.

4.2.9 Changes in Pre-treatment TroVax Response Biomarkers in Advanced Metastatic CRC Patients During Treatment

The effect of TroVax treatment on the levels of the identified markers of response was explored across treatment, and between responders/non-responders. This analysis was performed for CCL17 and MMP-7 across the trial (TD1 to TD106) in patients receiving TroVax monotherapy (Group 3).

As shown in Figure 4.18, TroVax non-responders have significantly lower pre-treatment (TD1) CCL17. Across time, from TD1 to TD106, the levels of CCL17 remain generally lower in non-responders than responders, although these differences were not statistically significant after TD1 (Figure 4.23A). In these TroVax non-responders, there was a significant increase in plasma CCL17 between TD1 and TD29/TD43. No change was seen in the TroVax responder group (Figure 4.23A). This trend remained the same when patients from Group 4, receiving dual CPM and TroVax treatment, were included in the analysis, with a significant increase in CCL17 also seen in non-responders between TD1 and TD29/43/78 (data not shown). There were no changes in plasma CCL17 across time for non-treated controls (data not shown).

It has also been previously shown that pre-treatment (TD1) levels of MMP-7 are significantly higher in TroVax non-responders (Figure 4.18). Throughout treatment, from TD1 to TD106, MMP-7 remained generally higher in TroVax non-responders than responders. This was significant at most time points (Figure 4.23B, significance not shown). There was no statistically significant change in the plasma level of MMP-7 over treatment between TD1 and any subsequent trial day, in either responders or non-responders. It does appear, however, that levels in non-responders increase over time (Figure 4.23B). This trend remained the same when patients from Group 4 were included in the analysis (data not shown). Plasma MMP-7 was not significantly altered over time in non-treated controls (date not shown).

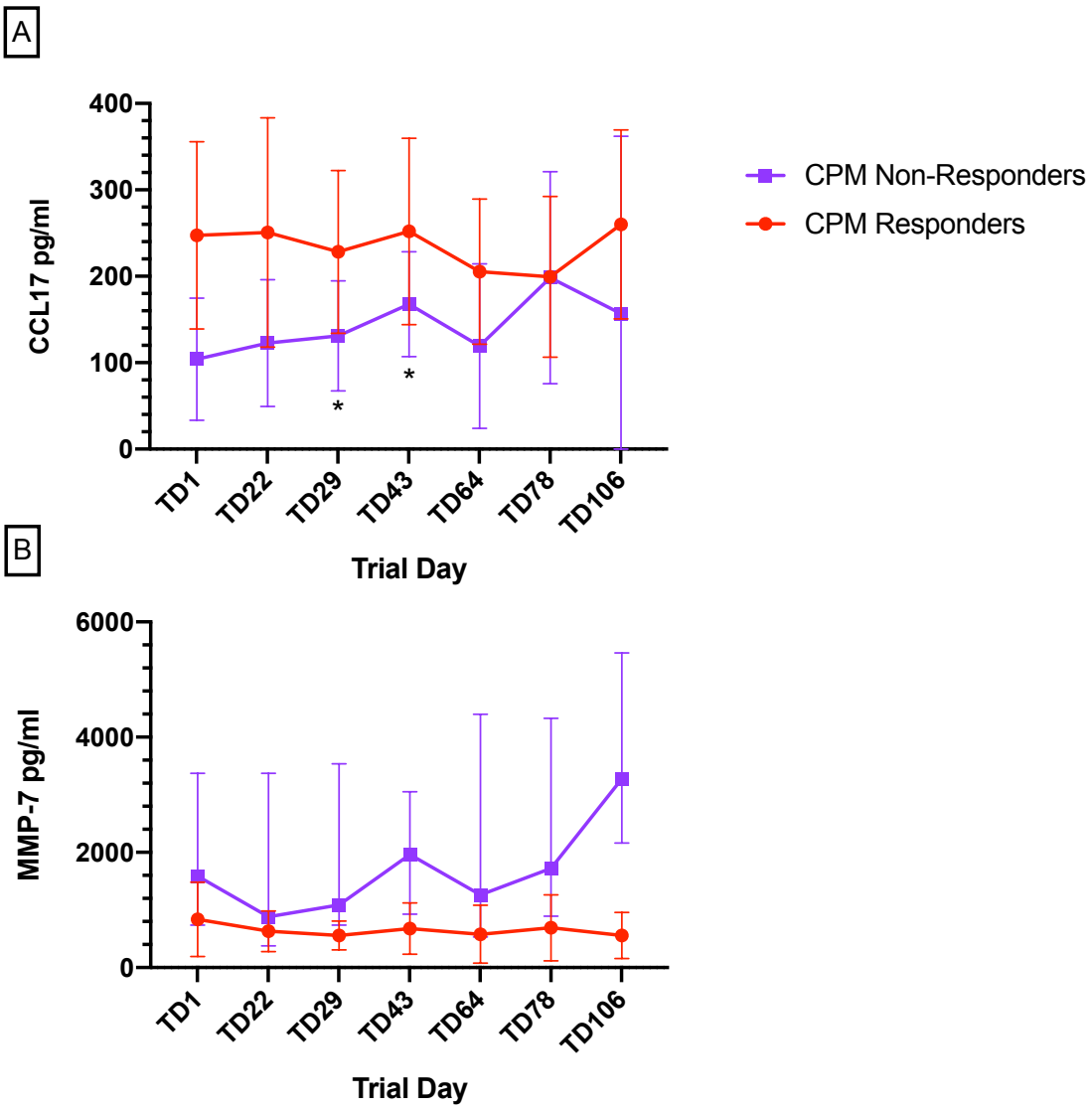


Figure 4.23 (A-B) Changes in plasma CCL17 and MMP-7 in mCRC patients across TroVax-treatment from TD1 to TD106.

TaCTiCC patients receiving TroVax monotherapy were split into those who responded to therapy (n=8-9) and those who did not respond to therapy (n=4-8). For each group, differences between TD1 and each subsequent trial day were compared using Wilcoxon signed-rank test. Mean with 95% confidence interval shown. Asterisk indications of significance are as per methods and indicate significant differences of the respective time point compared to TD1 of that group.

4.2.10 Association of Pre-Treatment TroVax Response Biomarkers with Patient Clinical Parameters

In order to investigate the physiological relevance of CCL17 and MMP-7 in advanced mCRC, their association with the following patient clinical parameters was explored:

1. Age
2. Sex
3. Primary Tumour Location
4. Site of Metastasis

CCL17 did not associate with any of the above clinical parameters. MMP-7, however, was significantly higher in patients with liver metastasis, when considering the entire TaCTiCC cohort (Figure 4.24A). As this result is potentially confounded by the presence of multiple sites of metastasis within several of these patients, the levels of MMP-7 were also considered in only those TaCTiCC patients with one site of metastasis. Using these criteria, higher MMP-7 levels were also demonstrated in patients with liver metastasis (Figure 4.24B).

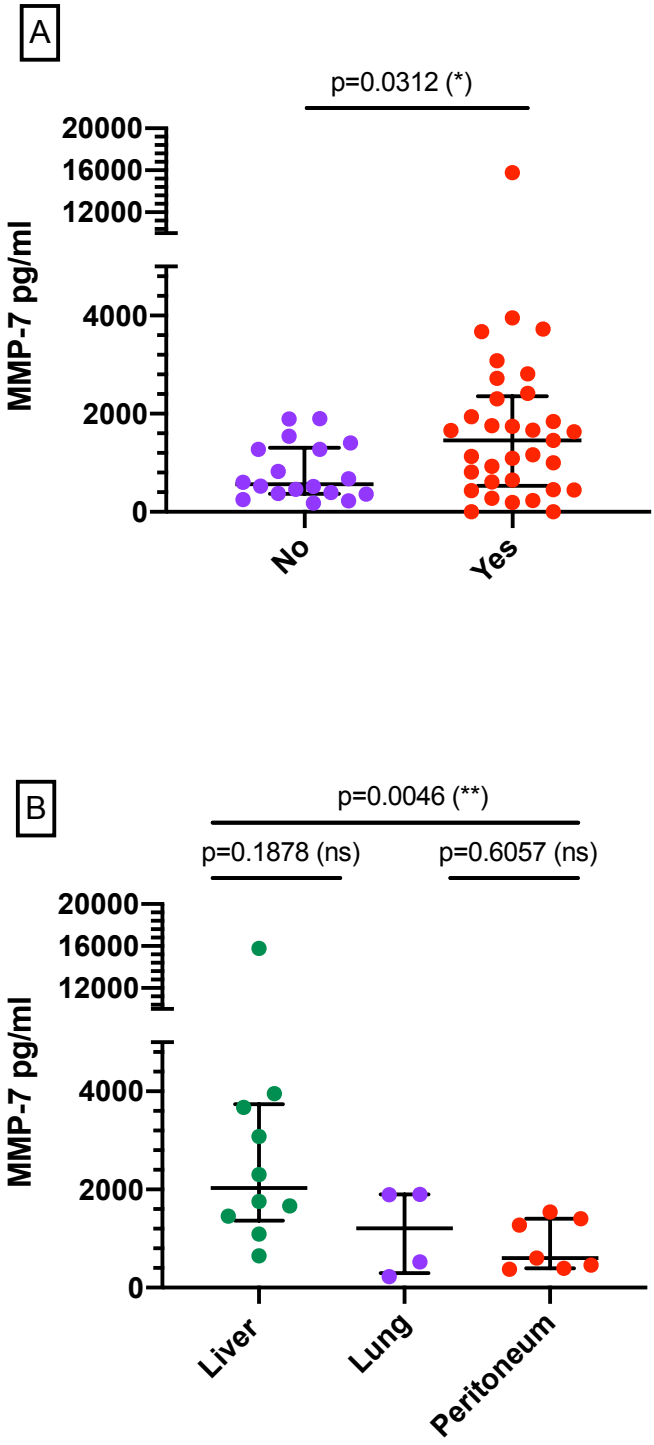


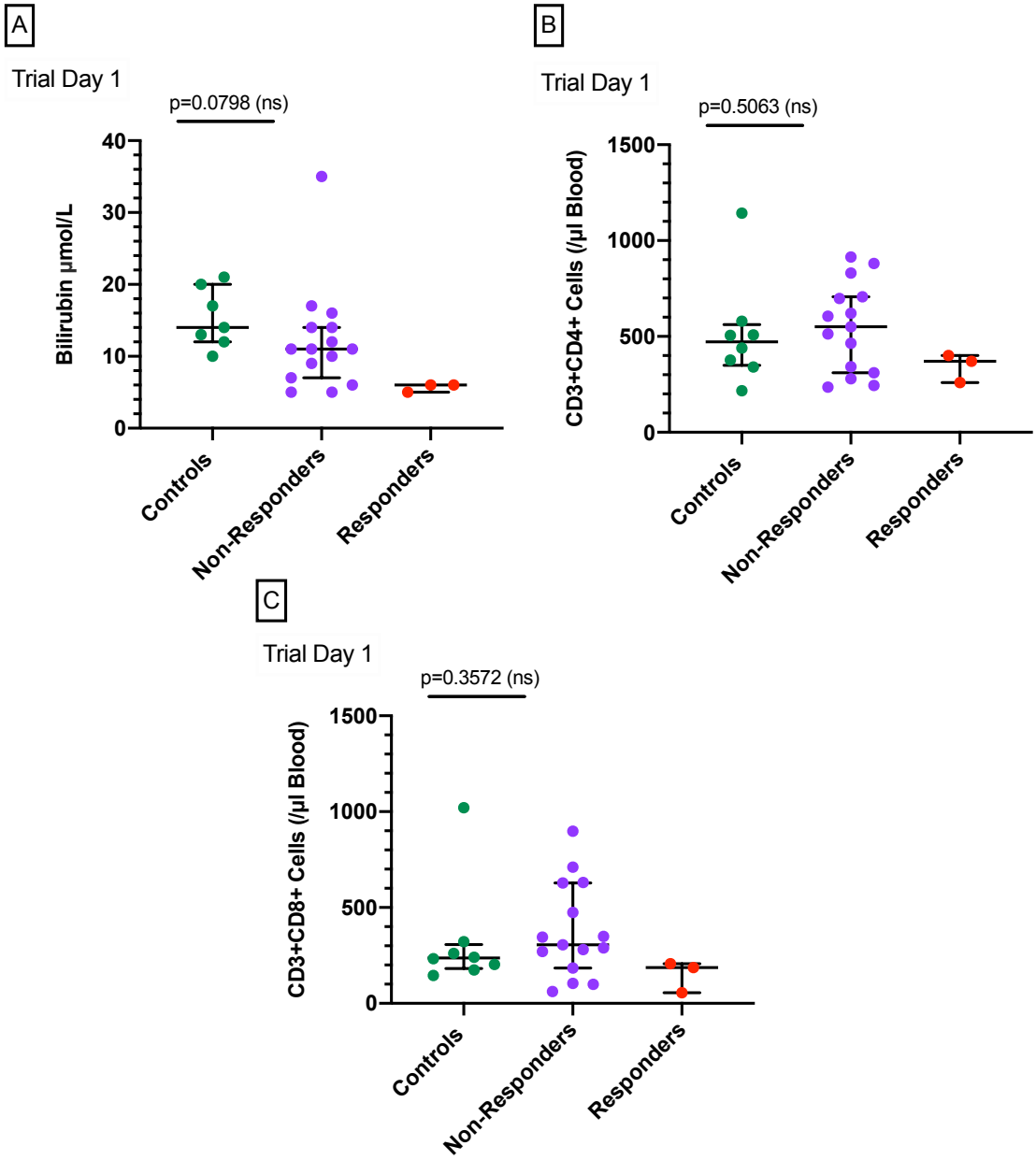
Figure 4.24 (A-B) Plasma MMP-7 is significantly higher in advanced mCRC patients with measurable liver metastasis.

Using either the entire TaCTiCC cohort (A, n=52), or only those TaCTiCC patients with one metastatic site (B, n=23), patients were split based on either presence of liver metastasis (A), or location of first metastasis (B).

4.2.11 Prediction of Advanced Metastatic CRC Patients Most Responsive to Dual Cyclophosphamide and TroVax Treatment Using Pre-Treatment Serological, Immunological and Plasma Protein Measurements

Several potential biomarkers of both CPM and TroVax response have been identified through this study. To understand the ability of such pre-treatment measurements to predict response to both therapies, patients from TaCTiCC group 4 receiving dual therapy were further investigated. These patients were stratified into responders/non-responders and pre-treatment serological, immunological, and plasma protein measurements, as per Sections 4.2.1 and 4.2.7, were compared between the groups. Patients were considered responders if they responded to CPM treatment based on both magnitude of T_{reg} depletion and IFNγ response to 5T4, and also responded to TroVax treatment (n=3). Non-responders were considered to meet either only one, or neither of these criteria (n=14-15). Given the small number of responders, it should be noted that the following results are intended only to guide future analysis.

Lower levels of bilirubin were found in treatment responders (Figure 4.25A). Responders also had significantly lower levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells (Figures 4.25B and 4.25C). Of the investigated plasma proteins, there were lower levels of BDNF, MMP-3, Tenascin-C, and TIMP-1 in treatment responders (Figures 4.25D, 4.25E, 4.25F, and 4.25G). Statistical analysis was not performed for comparisons of responders and non-responders due to the small group size of responders (n=3).


A
Trial Day 1
p=0.0798 (ns)
Bilirubin µmol/L
40
30
20
10
0
Controls
Non-Responders
Responders
B
Trial Day 1
p=0.5063 (ns)
CD3+CD4+ Cells (/µl Blood)
1500
1000
500
0
Controls
Non-Responders
Responders
C
Trial Day 1
p=0.3572 (ns)
CD3+CD8+ Cells (/µl Blood)
1500
1000
500
0
Controls
Non-Responders
Responders

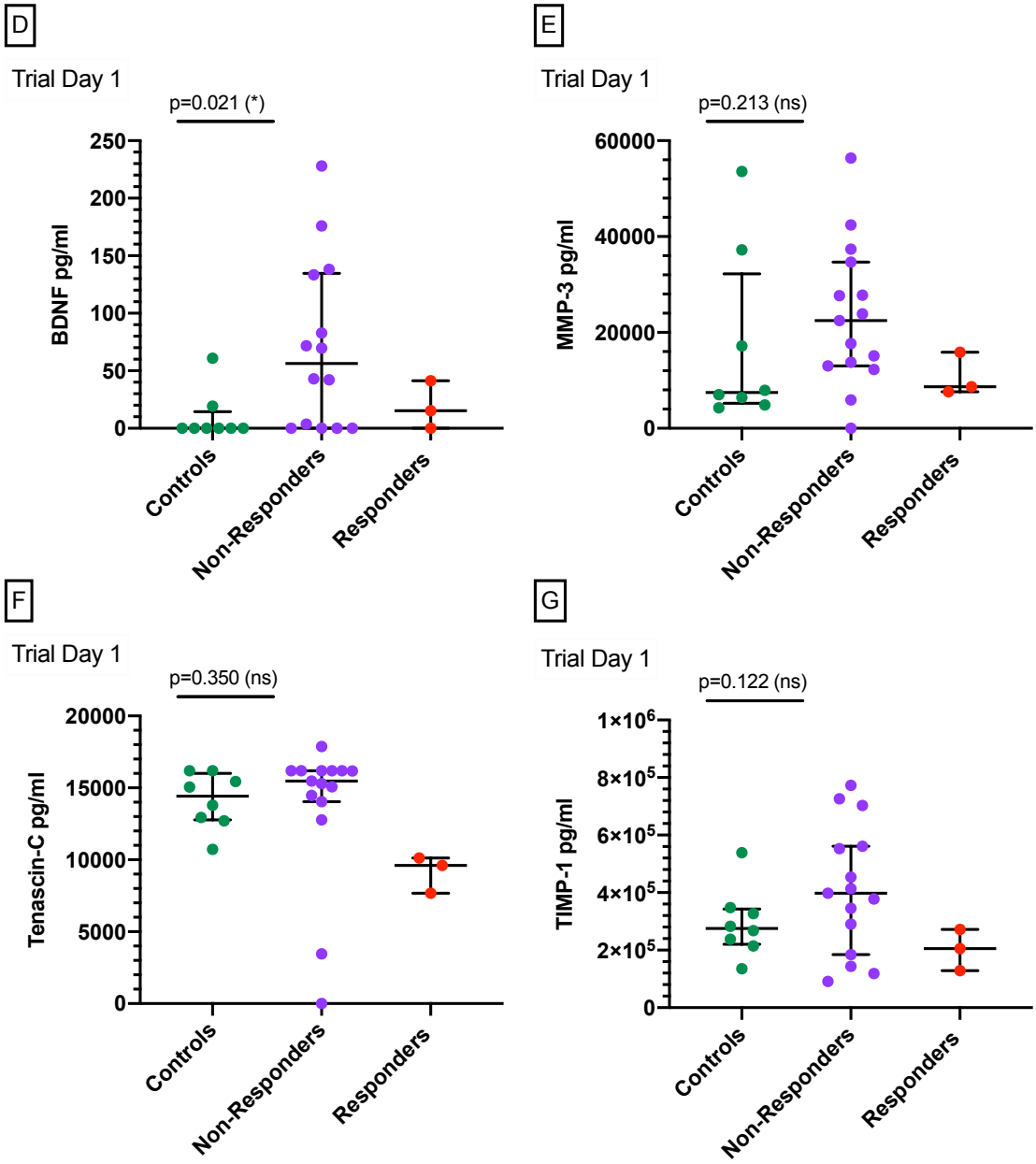


Figure 4.25 (A-G) Multiple immunological, serological, and plasma protein measurements are significantly altered in those most responsive to combined CPM and TroVax treatment.

Patients from patients TaCTiCC Group 4, receiving CPM and TroVax dual therapy, were split into those who responders to were most immunologically responsive to CPM treatment and responded to TroVax (n=3), compared to those who did not meet both of these criteria (n=14-15).

4.3 Discussion

The field of cancer immunotherapy has grown exponentially in the last decade and has undoubtedly proven revolutionary for the treatment of some cancers. To optimally treat patients, it is important that we can understand and predict those individuals most likely to benefit from a given therapy. This is particularly important considering the rapid pace new drugs and treatments are emerging, and the frequently exorbitant costs associated.

In this Chapter, the usefulness of pre-treatment blood measurements for the prediction of CPM and/or TroVax treatment response was described, specifically in the context of advanced mCRC patients from the TaCTiCC clinical trial. Previous serological and immunological measurements taken during the trial were assessed, as were a panel of plasma proteins retrospectively measured from frozen patient plasma samples. The results of this investigation identify potential biomarkers of response to these immunotherapies, warranting further investigation.

Extracellular Matrix (ECM) Proteins MMP-3, MMP-7 and TIMP-1 Can Predict Response to CPM and/or TroVax Immunotherapy

The results of the study indicate that pre-treatment plasma levels of MMP-3 and TIMP-1 are significantly higher in advanced mCRC patients who do not respond to CPM therapy. MMP-7 was also shown to be increased in patients who did not respond to TroVax immunotherapy. Matrix metalloproteinases (MMPs) and endogenous tissue inhibitors of metalloproteinases (TIMPs) play a role in many physiological processes, for example tissue remodelling. Conversely, their aberrant activity has been documented in a vast range of disease settings, for example cancer and arthritis.

MMP-3 and MMP-7 are involved in extracellular matrix cleavage and remodelling, which can occur in the context of tissue repair, and also in cancer cell invasion. In addition to this function, MMP-7 has been identified as a driver of angiogenesis (Ito *et al*. 2007; Ito *et al*. 2009), and both MMP-3 and MMP-7 have shown to cleave cell-surface E-cadherin, a process known to drive tumour progression and metastasis (Noë *et al*. 2001). Increased serum MMP-7 is associated with reduced overall survival in CRC patients (Maurel *et al*. 2007; Klupp *et al*. 2016), and increased serum MMP-3 is associated with decreased survival in ovarian cancer patients (Cymbaluk-Płoska *et al*. 2018). There is also evidence that increased tumoral expression associates with reduced survival, for example MMP-3 in breast, pancreatic and lung cancer (Mehner *et al*. 2015).

These metalloproteinases may therefore represent markers of overall poor survival in advanced mCRC patients, potentially explaining their increased expression in the plasma of patients who do not respond to therapy.

Previous studies have associated increased pre-operative plasma levels of TIMP-1 with poor survival outcomes in CRC (Holten-Andersen *et al*. 2000; Yukawa *et al*. 2004). Similar findings have also been described for tumour tissue levels of TIMP-1 in breast cancer patients (McCarthy *et al*. 1999; Schrohl *et al*. 2004). These findings are somewhat paradoxical as physiologically, TIMPs inhibit MMPs, and MMPs have been well-described as being increased in cancer and driving metastasis. Therefore, is would be reasonable to consider that an increased in TIMPs would be beneficial for cancer. TIMPs have additional roles, however, for example, TIMP-1 is able to act as a growth factor for a large range of cell types (Hayakawa *et al*. 1992), has been widely reported to inhibit cellular apoptosis (Guedez *et al.* 1998; Li *et al.* 1999; Murphy *et al*. 2002; Lee *et al*. 2003; Liu *et al.* 2003; Boulday *et al*. 2004; Liu *et al.* 2005), and has been linked to the accumulation of cancer-associated fibroblasts in CRC (Gong *et al*. 2013). Increased level of TIMP-1 within primary breast cancer tumours has been linked to poor response to first-line cyclophosphamide/methotrexate/5-fluorouracil (CMF) and anthracycline-based chemotherapy in metastatic breast cancer patients (Schrohl *et al*. 2006; Klintman *et al*. 2010). These chemotherapeutic agents work by inducing programmed cell death; therefore, it is possible that TIMP-1 impinges upon their function by preventing apoptosis. The effect of TIMP-1 on chemotherapy-induced apoptosis has previously been investigated in mouse fibrosarcoma cells. Compared to wild-type cells, those deficient in TIMP-1 showed increased response to standard chemotherapy drugs (Davidsen *et al*. 2006).

It is possible that increased levels of MMP-3, MMP-7, and TIMP-1 simply identify patients with increased disease progression or metastatic burden. Given the diverse roles employed by ECM proteins it is also possible, however, that these proteins, in particular TIMP-1, impinge upon the immunotherapeutic efficacy of CPM and/or TroVax.

ECM Proteins MMP-3, MMP-7, and TIMP-1 Associate with Liver Metastasis

MMP-3, MMP-7 and TIMP-1 were also significantly shown to be higher in patients with liver metastasis. This is strongly supported by the findings of several other researchers. MMP-3, for example, has been linked to CRC liver metastasis in murine cancer cell lines through a pathway involving Cyclin-dependent kinase 8 (CDK8) and WNT/β-catenin signalling (Firestein *et al*. 2008; Liang *et al*. 2018), and MMP-7 mRNA

and protein overexpression has been documented in CRC liver metastasis compared to patient-matched healthy liver tissue (Zeng *et al*. 2002). In addition, there is strong evidence supporting a role of TIMP-1 in CRC liver metastasis. Overexpression of TIMP-1 mRNA has been shown in CRC tumours and liver metastases compared to patient-matched normal mucosa and liver (Zeng and Guillem 1995), and increased plasma TIMP-1 has been identified as a prognostic factor in CRC patients with liver metastasis (Bunatova *et al*. 2012). After resection of liver metastasis, CRC patients with high pre-surgery serum TIMP-1 were found to have increased risk of liver metastasis recurrence (Min *et al*. 2012). Interesting, a study has also shown that CRC patients with liver metastasis have worse responses to palliative 5-FU chemotherapy when liver metastases have increased MMP-7 and TIMP-1 (Gentner *et al*. 2009).

In murine models, it has been proposed that TIMP-1 may act as a negative regulator of hepatocyte growth factor (HGF) during liver regeneration; mice with TIMP-1 loss of function showed increased hepatocyte cell division (Mohammed *et al*. 2005). HGF is involved in the progression of hepatocytes through the cell cycle, an important process for liver regeneration. This is further supported by a study which showed that increased stromal TIMP-1 led to induction of HGF signalling, promoting liver metastasis (Kopitz *et al*. 2007). In mouse models, plasma TIMP-1 has also been implicated in the formation of the premetastatic niche within the liver through increased recruitment of neutrophils via SDF-1 (Seubert *et al*. 2015).

The above lines of evidence support the finding of this thesis that MMP-3, MMP-7, and TIMP-1 associate with the presence of liver metastasis in CRC patients, and suggest plausible mechanisms of action. Interestingly, therapeutic effects of CPM are reliant upon metabolization to active metabolites within the liver by cytochrome p450 enzymes (Struck *et al*. 1987; Crespi *et al*. 1993; Moore 2005). It could be hypothesised that liver metastasis would alter the expression of such enzymes, directly impacting the availability of active CPM metabolites; this may reduce the efficacy of CPM treatment. In hepatocellular carcinoma (HCC), the expression profile and activity of cytochrome p450 enzymes is altered compared to non-cancer controls (Lane *et al*. 2004). This was also described in patients with severe chronic liver disease (George *et al*. 1995). It is, therefore, plausible that metastasis-associated liver damage may alter the expression of cytochrome p450 enzymes required for CPM metabolism.

Pre-Treatment Immune Cell Numbers and Immunological Plasma Proteins Determine Response to CPM and/or TroVax Immunotherapy

A reduction in several immunological measurements, namely basophil, $CD3^+CD4^+$, T_{reg} and WBC number, was demonstrated in responders to CPM immunotherapy. Increased plasma CCL17 and decreased IFNγ 5T4 T cell response were also identified in TroVax responders. These results suggest that the pre-treatment immunological landscape is different between individuals who will respond to immunotherapy, and those who will not.

In the context of TroVax it stands to reason that individuals who will benefit most from treatment are those patients with low level or no pre-existing T cell responses to 5T4. In patients who already have high circulating responses, there would be no clear or rational advantage to increasing the response. CCL17 is a cytokine involved in T cell chemotaxis through its interaction with chemokine receptor 4 (CCR4) (Imai *et al*. 1997). Serum CCL17 has been associated with improved survival outcomes in patients with advanced melanoma (Weide *et al*. 2015). A previous study investigating the effect of CPM and multipeptide cancer vaccination (IMA901) on survival in renal cell carcinoma (RCC) identified increased serum levels of CCL17 in patients who demonstrated multipeptide and immune responses. The study also found high serum CCL17 associated with increased overall survival in patients receiving CPM as part of their therapy (Walter *et al*. 2012). The findings described in this Chapter are the first to show the association of increased plasma CCL17 with positive immune response to TroVax in CRC patients.

Pre-treatment differences in immune cell numbers between CPM responders and no-responders were described in this Chapter. It is well known that in addition to other markers, T_{reg} cells express CD3 and CD4. It is therefore likely that the difference in $CD3^+CD4^+$ cell number between responders/non-responders is also identifying differences in T_{reg} cells. CPM is able to selectively deplete T_{reg} cells, therefore the increased prevalence of T_{regs} in the PBMC of CPM non-responders may represent a barrier for treatment efficacy; perhaps a higher dose of CPM would be able to deplete a higher number of these cells. In addition to increased, T_{reg} cell number, WBC count was also increased in non-responders. WBC count has been linked to inflammation, which is known to be involved in the development of CRC. It has been shown, for example, that increased WBC count in CRC patients is associated within increased risk of mortality (Lee *et al*. 2006). Collectively, these changes highlight the importance of pre-treatment

immune function on immunotherapy outcome. These measurements are taken routinely by the NHS, therefore represent a powerful and free resource for further investigation.

Limitations

Although the results presented here have identified several potential response biomarkers to CPM and TroVax immunotherapy, this investigation was primarily intended to identify factors of interest for future investigation. Given that the TaCTiCC clinical trial was performed in small cohort of patients (n=52) across four treatment groups, the results presented are limited by the number of observations. It is therefore vital that these results are validated in a larger cohort of patients. Additionally, some results were subject to technical difficulty, for example the levels of Tenascin-C were often above the upper limit of detection. In future analyses, it would be advisable to optimise plasma dilutions in-house for the patient cohort. Luminex Multiplex is a comparatively expensive technique, and as a result, the plasma proteins investigated were chosen based on previous associations with CRC, and from the results of small pilot experiments. They do not, therefore, represent an exhaustive analysis of all potential markers of response.

Future Directions

TaCTiCC demonstrated a significant survival benefit associated with the treatment of advanced mCRC patients with low-dose metronomic CPM. Treatment depleted regulatory T cells and promoted 5T4-specific T cell responses. This is striking evidence that CPM treatment can promote anti-tumour immunity. This drug is cost-effective, readily available, and is safe, with minimal side effects.

The results of the described study indicate that patients with higher levels of several ECM proteins are less likely to respond to CPM treatment. These proteins are known to be instrumental in the progression and metastasis of cancer. It has also been demonstrated that several immunological measurements of interest for predicting response to CPM therapy. Similar markers were shown to be useful in identifying patients who responded to combined CPM and TroVax treatment, however the group size was limited. It is well documented that cancer progression is associated with several immunological changes, for example an increase in T_{reg} frequency in peripheral blood lymphocytes (Ling *et al*. 2007), and a decline in anti-tumour immune responses (Scurr *et al*. 2013). It could therefore be reasonably argued that patients with factors associating with more advanced and aggressive disease, for example increased ECM proteins and

altered immunological parameters, are less likely to respond to immunotherapeutic CPM. This, in combination with the TaCTiCC survival data, provide clear rationale to investigate the effect of low dose metronomic CPM on anti-tumour immunity and disease-free survival in earlier stage CRC patients.

An investigation into the effect of CPM treatment on earlier-stage CRC patients is planned within the Godkin/Gallimore group. This will be completed immediately after conventional therapy, for example surgical resection and adjuvant chemotherapy. In addition to assessing the benefit of targeting earlier stage CRC patients with CPM immunotherapy, this trial will allow the validation of identified markers of CPM response. Moreover, it will provide access to additional patient samples in order to identify other potential biomarkers of response within this less advanced cohort.

In addition to investigating the identified CPM response biomarkers in a further patient cohort, it would be interesting to investigate the physiological relevance of the identified matrix proteins in CRC. It has been shown that TIMP-1, MMP-3 and MMP-7 are higher in the plasma of patients with liver metastasis. Additionally, the results described in Chapter 3 indicated that MMP-3 and TIMP-1 are significantly higher in mCRC patients than non-metastatic CRC patients. For TIMP-1, the levels demonstrated in non-metastatic CRC patients were also significantly higher than those found in non-cancer controls. It would be interesting to further investigate the association of these matrix proteins to the presence of CRC, and subsequent metastasis, particularly within the liver. This could be achieved by measuring the levels of these proteins in a range of CRC patients prior to surgical intervention, and comparing based on presence and location of metastasis, and to a group of non-cancer controls. The size of identified metastatic liver lesions could be correlated with the protein levels to further explore their potential for the identification of site-specific metastasis and early diagnosis. Furthermore, it would be interesting to investigate the association of CRC liver metastasis on the expression profile of cytochrome p450 enzymes, and subsequent metabolization of CPM. This could be performed in existing in-house mouse models of CRC undergoing CPM treatment.

While the results of this study have identified several potential biomarkers of treatment response for both CPM and TroVax immunotherapy in advanced mCRC patients, they imply the need for earlier immunotherapeutic intervention. The changes associated with non-response to therapy also associate with cancer progression and/or aggression. The usefulness of these response markers in earlier stage patients is therefore uncertain.

4.4 Key Findings

Pre-treatment plasma proteins, particularly ECM proteins, and immunological measurements are significantly altered between mCRC patients who respond to CPM/TroVax immunotherapy and those who do not. These represent potential response biomarkers and warrant investigation in a future patient cohort. Moreover, they highlight that the extent of cancer metastasis and associated immunological change may influence response to immunotherapy. Therapeutic outcomes may be improved by targeting patients earlier.

Predicting Cyclophosphamide Response

1. Advanced mCRC patients who responded to CPM treatment had significantly lower pre-treatment plasma levels of ECM proteins MMP-3 and TIMP-1. Higher pre-treatment levels of both proteins also associated with presence of liver metastasis, and increased pre-treatment TIMP-1 significantly associated with reduced overall and progression-free survival. Levels of these proteins were unaffected by CPM and remained generally altered between responders and non-responders throughout treatment.

2. Patients who responded to CPM treatment also had lower levels of basophils, $CD3^+CD4^+$ cells, T_{reg} cells, and WBCs. Higher pre-treatment $CD3^+CD4^+$ and T_{reg} cells associated with right-handed primary tumours, patients without peritoneal metastasis, and, in CPM-treated patients, reduced progression-free survival. Levels of $CD3^+CD4^+$ and T_{reg} cells were reduced by CPM treatment, however remained generally lower in responders throughout treatment.

3. Combining several CPM response biomarkers increases sensitivity and specificity of prediction. This should be implemented in future biomarker research.

Predicting TroVax Response

1. Advanced mCRC patients who responded to TroVax treatment had significantly higher pre-treatment levels of plasma CCL17 and lower levels of plasma MMP-7, although changes were not quite significant at TD22, immediately prior to TroVax injection. Increased pre-treatment MMP-7 also associated with the presence of liver metastasis and reduced overall and progression-free survival.

Levels of MMP-7 were unaffected by TroVax treatment and remained generally higher in non-responders. CCL17 was significantly increased in non-responders during TroVax treatment, however remained generally higher in responders.

Identifying the Most Immunologically Responsive Patients to Dual CPM/TroVax Immunotherapy

1. The most immunologically responsive patients to CPM/TroVax immunotherapy had reduced levels of several ECM proteins, including MMP-3, Tenascin-C, and TIMP-1. Additionally, they had lower levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells

2. Although in a small number of patients, these results suggest that advanced mCRC patients with less advanced disease may respond best to immunotherapy. The efficacy of immunotherapy may be increased by targeting patients earlier.

Chapter 5 Exploring the Immunogenicity of Novel Tumour Antigens in Healthy Controls and CRC Patients

5.1 Introduction

Cancer vaccination has proven relatively ineffective in patients with advanced disease. A clear example of this is the TaCTiCC clinical trial, where although survival benefits were demonstrated in patients who responded to 5T4 vaccination, all patients progressed within 10 months (Scurr *et al.* 2017a). The reasons for this lack of therapeutic efficacy may relate to increased immunosuppression in advanced cancer, however, may also be the result of poor antigenic targets.

Recently, efforts have been made within the Godkin/Gallimore group to identify novel antigen targets for CRC. Through epithelial cell adhesion molecule (EpCam) purification of cancer cells and subsequent RNA sequencing of tissue from 3 CRC patients, 7 candidate antigens were identified. These candidates were significantly different in tumour tissue of at least 2/3 patients, compared to paired proximal and distal epithelial samples from normal bowel. The following candidates were identified:

1. ARSJ (Arylsulfatase Family Member J)
2. CEACAM3 (Carcinoembryonic Antigen Related Cell Adhesion Molecule 3)
3. CENPQ (Centromere Protein Q)
4. CYP2B6 (Cytochrome P450 Family 2 Subfamily B Member 6)
5. DNAJB7 (DNAJ Heat Shock Protein Family (Hsp40) Member B7)
6. ZC3H12B (Zinc Finger CCCH Domain-Containing Protein 12B)
7. ZSWIM1 (Zinc Finger SWIM Domain-Containing Protein 1)

It was hypothesised that, similar to other tumour antigens e.g. oncofoetal antigen 5T4, that these candidates would be immunogenic, and that the extent of immunogenicity would be altered between healthy donors and CRC patients. The TaCTiCC clinical trial clearly demonstrated increased IFNγ responses to 5T4 in CRC patients receiving CPM treatment, particularly in those patients responding to therapy. It was therefore also hypothesised that CPM treatment would increase immune responses to the identified novel tumour antigens.

This Chapter investigates the immunogenicity of the identified candidate novel tumour antigens in the PBMC of healthy donors and CRC patients, both *ex vivo* and in

cultured cell lines, using pools of overlapping 20mer peptides. The effect of CPM treatment on cultured T cell responses to these candidates is also explored in the context of the TaCTiCC clinical trial.

5.2 Results

5.2.1 Ex vivo T Cell Responses to Novel Tumour and Control Antigens in Healthy Donors

To validate the use of Immunospot assays for measuring *ex vivo* T cell responses to antigens, IFNγ T cell responses to the following control antigens were measured in 6 healthy donors:

1. Purified protein derivative of tuberculin (PPD)
2. Influenza hemagglutinin (HA)
3. Tetanus toxoid (TT)
4. Phytohemagglutinin (PHA)

Similar *ex vivo* T cell responses were demonstrated in all healthy donors (Figure 5.1). A representative example of the ELISpot assay is shown in Figure 5.2A for 2 donors. For every *ex vivo* ImmunoSpot experiment shown, background activation has been subtracted from each response. This is the number of spots seen in when PBMC are plated without antigen stimulation and varies between individuals. To investigate whether *ex vivo* T cell responses are altered in the same individual over time, this assay was repeated in one donor at three time points; initial measurement, +2 weeks, +14 weeks. There are natural fluctuations in antigen responsiveness (Figure 5.3).

It is possible to measure the release of additional analytes through the use of FluoroSpot as opposed to ELISpot. To maximise the information obtained, remaining experiments were performed using IFNγ and Granzyme B (GZMB) FluoroSpot, allowing both Th1 and GZMB-mediated cytotoxic T cell responses to be investigated. This allowed an assessment of CD4/CD8 T cell responses to the identified novel tumour antigen candidates. A representative example of this FluoroSpot assay for control antigens in a healthy donor is shown in Figure 5.2B.

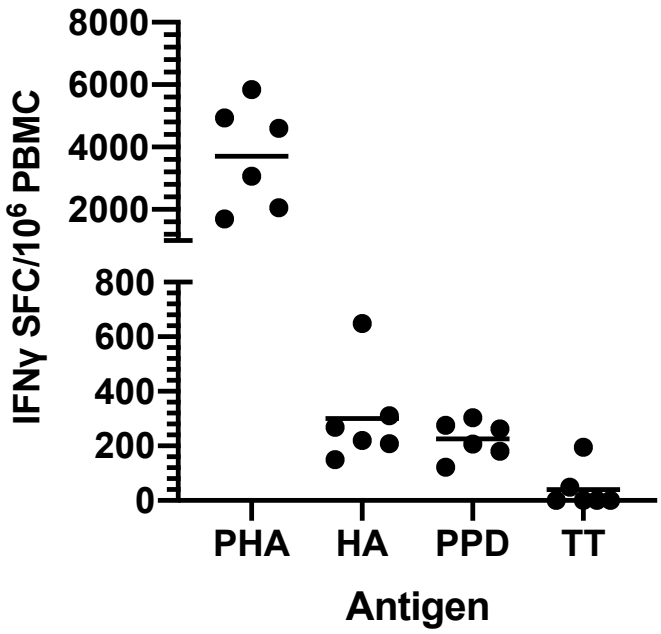


Figure 5.1 Measurement of *ex vivo* IFNγ T cell responses to control antigens in healthy donors.

Ex vivo IFNγ T cell responses to controls antigens PPD, HA, TT, and PHA were measured in triplicate using freshly isolated PBMC from 6 healthy donors. 2.5 $x10^5$ PBMC were plated per well. Mean response per antigen, normalised to spot forming cells per 10^6 PBMC, is shown for each donor. Background responses have been subtracted.

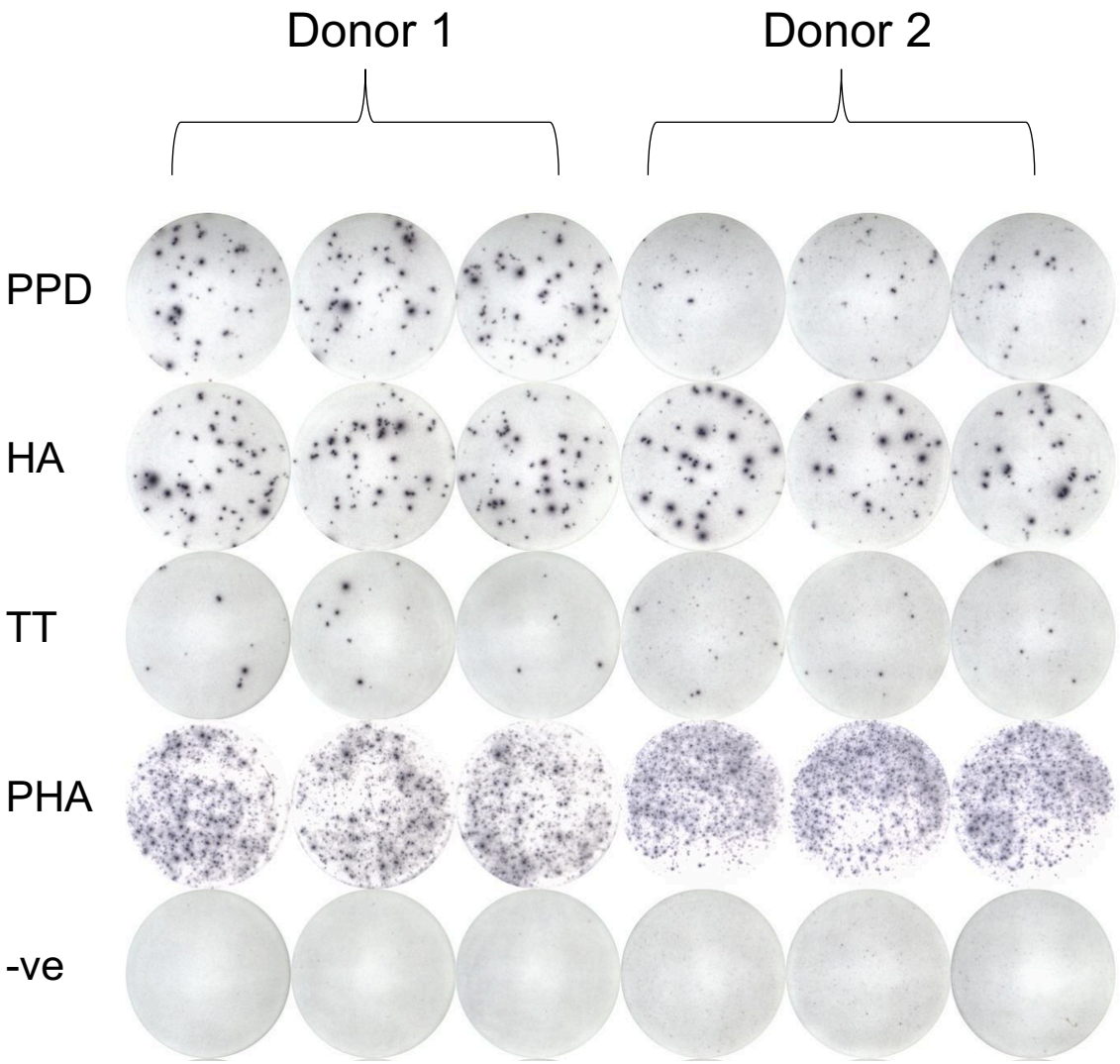


Figure 5.2 (A) Representative example of healthy donor *ex vivo* IFNγ ELISpot to control antigens.

Ex vivo IFNγ T cell responses to controls antigens PPD, HA, TT, and PHA were measured in triplicate. 2.5 x10^5 freshly isolated PBMC per well, from 2 healthy donors. Negative control wells without antigen also shown.

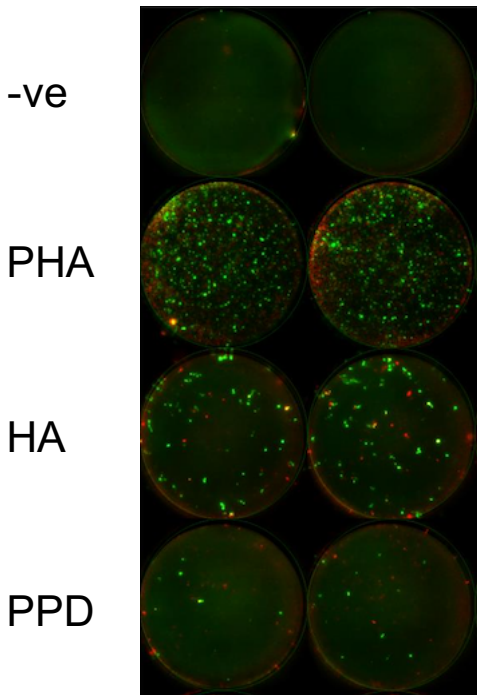


Figure 5.2 (B) Representative example of healthy donor *ex vivo* IFNγ/GZBM FluoroSpot to control antigens.

Ex vivo IFNγ/GZMB T cell responses to controls antigens PPD, HA, and PHA were measured in duplicate. 2.5 x10^5 freshly isolated PBMC per well, from 1 healthy donor. Negative control wells without antigen also shown. IFNγ shown in green, GZMB shown in red.

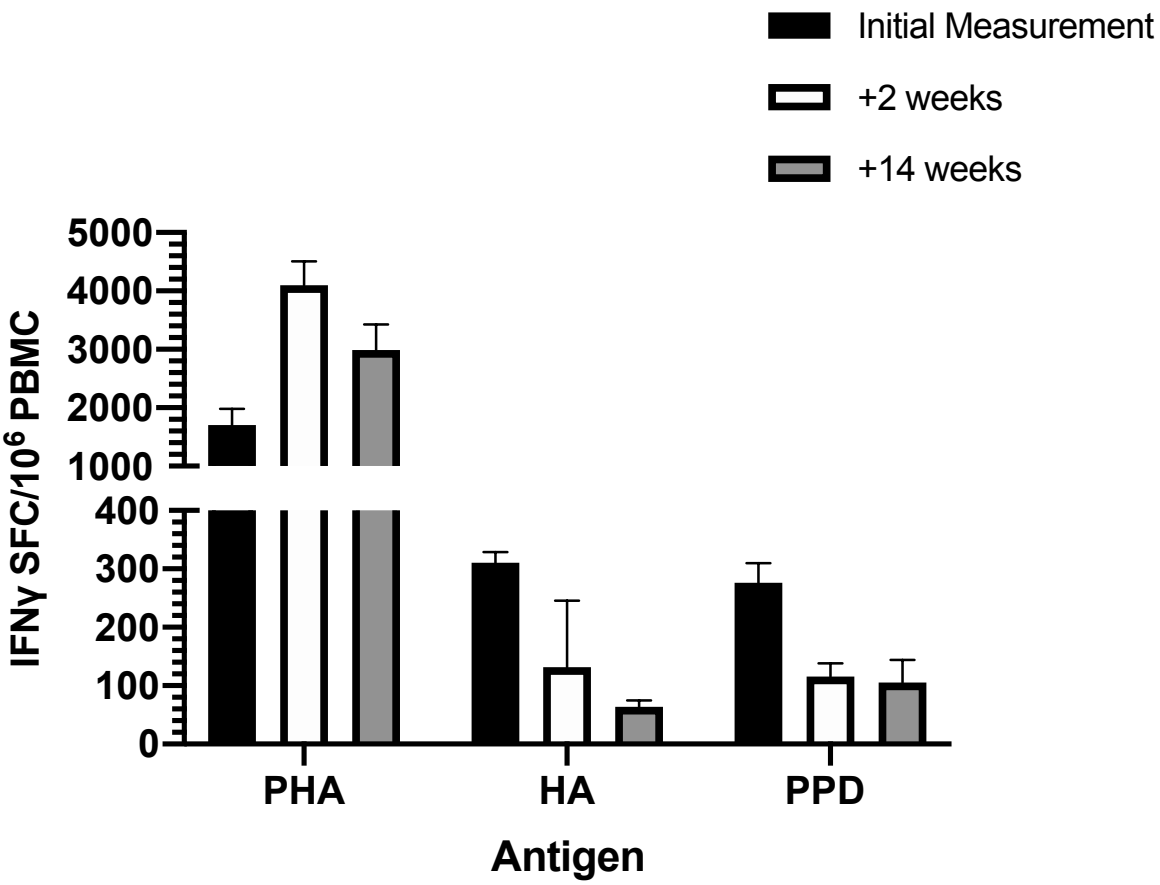


Figure 5.3 There are differences in *ex vivo* IFNγ T cell responses to control antigens healthy controls over time.

Ex vivo IFNγ T cell responses to controls antigens PPD, HA, TT, and PHA were measured in one healthy donor at three timepoints; initial measurement, +2 weeks, and +14 weeks. Average of three triplicate measurements shown normalised to SFC/10^6 PBMC, plus standard deviation (SD).

Ex vivo IFNγ/GZMB FluoroSpot assays were performed using freshly isolated PBMC from 11 healthy donors. Responsiveness to each antigen peptide pool (2-3 pools, as detailed in Methods) was measured in replicates of 2-4, depending on available PBMC number. Overall response to each peptide pool was calculated by averaging these repeated measures and subtracting any background in control wells without antigen. Total magnitude of response to each antigen was calculated as an average of the combined response to each peptide pool.

Ex vivo IFNγ T cell responses to control antigens PHA, HA, and PPD were demonstrated across most donors (Figure 5.4A). Similarly, there were clear IFNγ 5T4 T cell responses in 10/11 donors. Responses to both CYP2B6 and ZSWIM1 were comparable to those seen for 5T4. Of the other potential novel tumour antigens, IFNγ T cell responses were seen in some donors, with the exception of ARSJ.

Ex vivo GZMB T cell responses were demonstrated in all donors for control antigen PHA (Figure 5.4B). Some donors also had responses to HA, and most had responses to PPD. Only 3 donors had GZMB T cell responses to tumour antigen 5T4. A high number of donors, however, had responses to novel antigen candidates CEACAM3, DNAJB7, and CYP2B6 (7, 5, and 11 donors respectively). Conversely, GZMB T cell responses to ARSJ, ZC3H13B, CENPQ, and ZSWIM1 were only demonstrated in 1-2 donors.

Of the 11 healthy donors, 7/11 were aged between 23-29, and 4/11 were aged 35-48. *Ex vivo* IFNγ and GZMB T cell responses to the aforementioned antigens were compared between these groups, and no significant differences were found (data not shown).

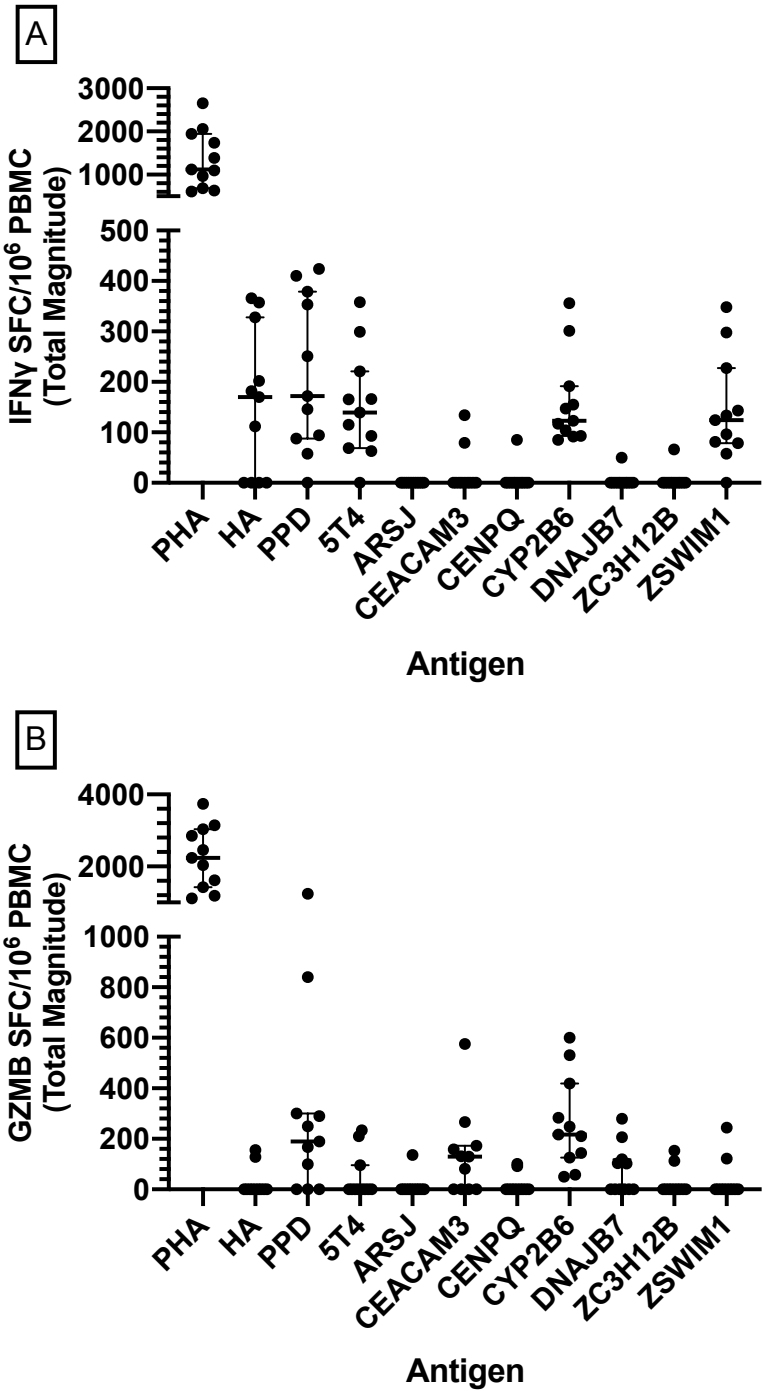


Figure 5.4 (A-B) *Ex vivo* IFNγ and GZMB T cell responses to control antigens, tumour antigen 5T4, and novel tumour antigen candidates in healthy donors.

Ex vivo IFNγ (A) and GZMB (B) T cell responses to control antigens PHA, HA, and PPD, CRC tumour antigen 5T4, and the 7 identified novel tumour antigen candidates were measured in freshly isolated PBMC from 11 healthy donors. 2.5 x10^5 PBMC were plated per well. Magnitude of response to each antigen, normalised to spot forming cells per 10^6 PBMC, is shown for each donor. Background responses have been subtracted. Median and interquartile range shown.

5.2.2 Ex vivo T Cell Responses to Novel Tumour and Control Antigens in Colorectal Cancer Patients

In order to investigate the responsiveness of CRC patients to the identified novel tumour antigen candidates, *ex vivo* IFNγ/GZMB FluoroSpot assays were performed for 4 patients; 2 patients with Dukes' stage B disease, 1 patient with Dukes' stage C disease, and 1 patient with Dukes' stage D disease. Responses were measured in duplicate, and magnitude of response was calculated as detailed in Section 5.2.1.

Ex vivo IFNγ T cell responses to PHA were demonstrated in all patients, whereas responses to HA and PPD were found in only a proportion of patients (Figure 5.5A). *Ex vivo* IFNγ T cell responses to 5T4 were only measurable in 1 patient. With regards to the novel tumour antigen candidates, IFNγ T cell responses to CYP2B6 were found in 3/3 patients, and 2/3 patients also had ZSWIM1 responses. No *ex vivo* IFNγ T cell responses were found for ARSJ, CEACAM3, CENPQ, DNAJB7, or ZC3H12B.

Ex vivo GZMB T cell responses were demonstrated to PHA in all patients (Figure 5.5B). No patients produced *ex vivo* GZMB T cell responses to either HA or 5T4, and only one patient mounted a response to PPD. *Ex vivo* GZMB T cell responses to ARSJ, CEACAM3, ZC3H12B, and ZSWIM were demonstrated in some patients, and responses to CYP2B6 were found in all patients. There was no measurable *ex vivo* GZMB T cell response to either CENPQ or DNAJB7. With the exception of PHA stimulation, no dual IFNγ/GZMB T cell responses were identified (data not shown).

T cell responses were also measured in one patient with a previous polyp adenoma, aged 31, undergoing a preventative subtotal colectomy. Bloods were taken prior to operation, and no retrospective evidence of current dysplasia or malignancy was found. *Ex vivo* IFNγ T cell responses were demonstrated for control antigen PHA, known tumour antigen 5T4, in addition to novel tumour antigen candidates CYP2B6 and ZSWIM1 (Figure 5.6 A&B). *Ex vivo* GZMB T cell responses were demonstrated for control antigen HA, and novel tumour antigen candidates ARSJ, CEACAM3, CYP2B6, ZC3H12B, and ZSWIM1. No dual responses were demonstrated (data not shown).

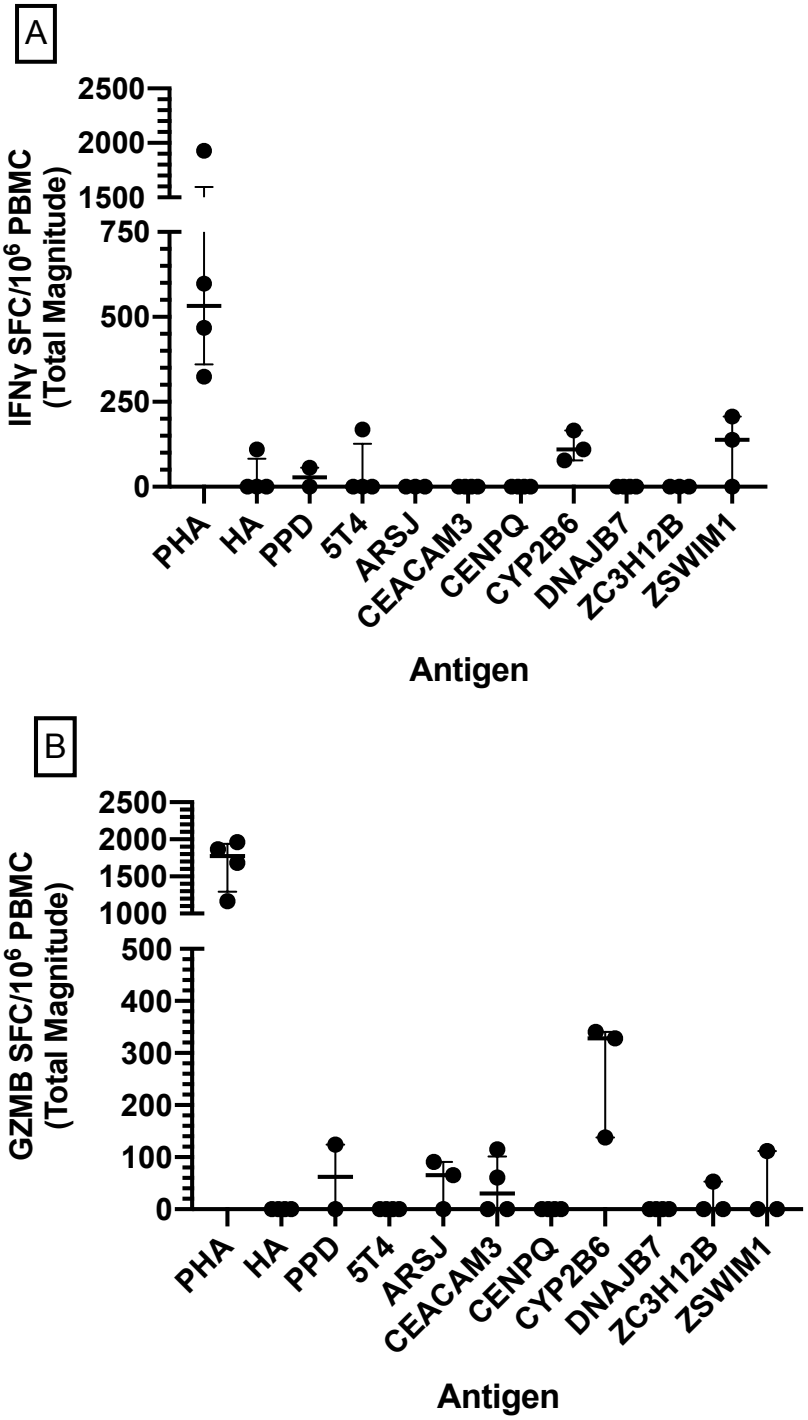


Figure 5.5 (A-B) *Ex vivo* IFNγ and GZMB T cell responses to control antigens, tumour antigen 5T4, and novel tumour antigen candidates in CRC patients.

Ex vivo IFNγ (A) and GZMB (B) T cell responses to control antigens PHA, HA, and PPD, CRC tumour antigen 5T4, and the 7 identified novel tumour antigen candidates were measured in freshly isolated PBMC from 3-4 CRC patients. 2.5 x10^5 PBMC were plated per well. Magnitude of response to each antigen, normalised to spot forming cells per 10^6 PBMC, is shown for each donor. Background responses have been subtracted. Median and interquartile range shown.

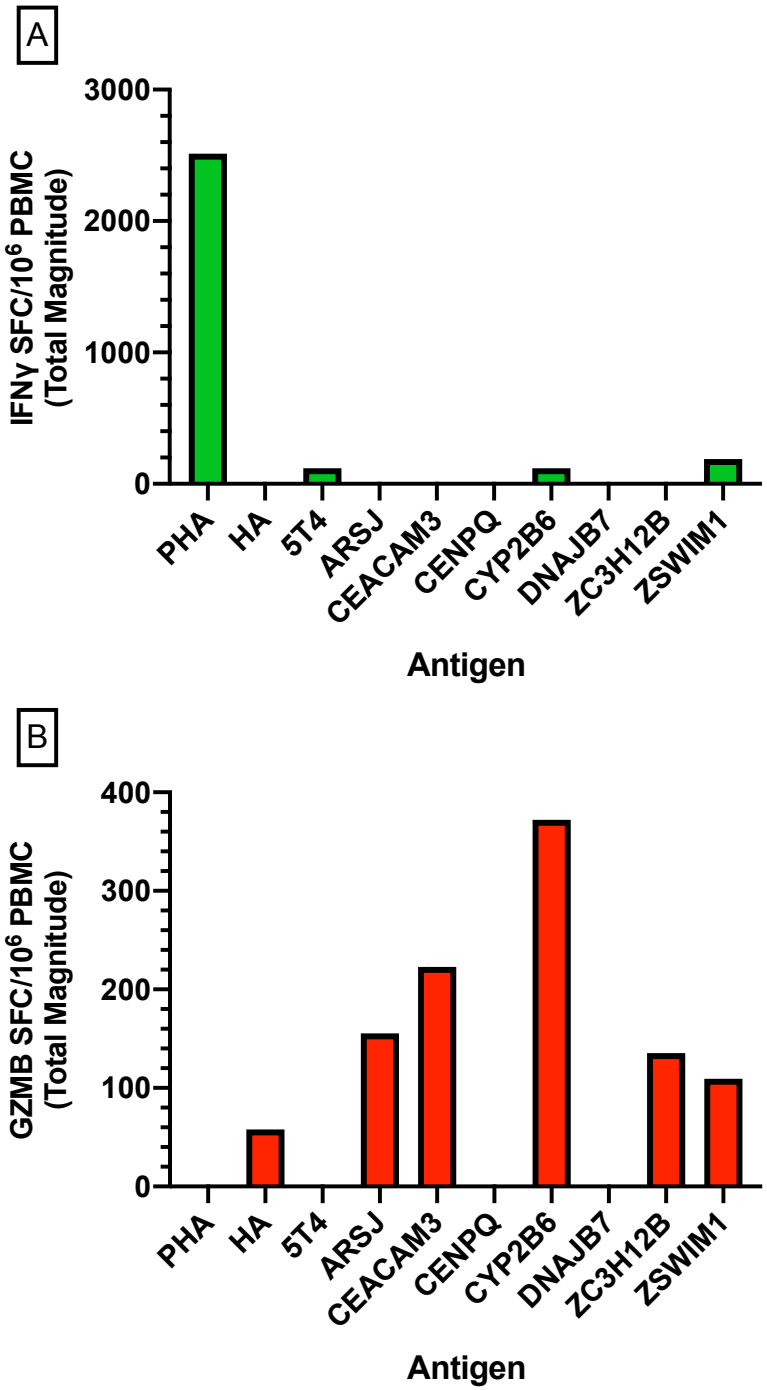


Figure 5.6 (A-B) *Ex vivo* IFNγ and GZMB T cell responses to control antigens, tumour antigen 5T4, and novel tumour antigen candidates in a patient with a previous polyp adenoma.

Ex vivo IFNγ (A) and GZMB (B) T cell responses to control antigens PHA, and HA, CRC tumour antigen 5T4, and the 7 identified candidate tumour antigens were measured in freshly isolated PBMC from 1 patient with a previous polyp adenoma undergoing a preventative subtotal colectomy. There was no evidence of dysplasia or malignancy at the time of PBMC isolation. 2.5 x10^5 PBMC were plated per well. Magnitude of response to each antigen, normalised to spot forming cells per 10^6 PBMC, is shown. Background responses have been subtracted.

5.2.3 Comparison of Ex vivo T Cell Responses to Novel Tumour and Control Antigens in Colorectal Cancer Patients and Healthy Donors

In Sections 5.2.1 and 5.2.2 *ex vivo* IFNγ and GZMB T cell responses to control antigens, known tumour antigen 5T4, and the identified novel tumour antigens candidates were investigated in healthy donors and CRC patients respectively. It is known that 5T4 T cell responses are significantly reduced in CRC patients than healthy controls (Scurr *et al*. 2013), therefore it was hypothesised that T cell responses to the novel candidates would also be altered between these groups.

When *ex vivo* IFNγ and GZMB T cell responses to control antigens PHA, HA, and PPD were compared between healthy donors and CRC patients, there were no significant differences (Figure 5.7 A&B). There was, however, a trend for increased magnitude of response to these antigens in healthy donors. *Ex vivo* IFNγ T cell responses to 5T4 were increased in healthy donors, although this did not reach statistical significance (Figure 5.8A). There were no clear differences between *ex vivo* IFNγ and GZMB T cell responses to the candidate tumour antigen candidates, however, as per the control antigens, there were generally higher responses in healthy donors (Figure 5.8B).

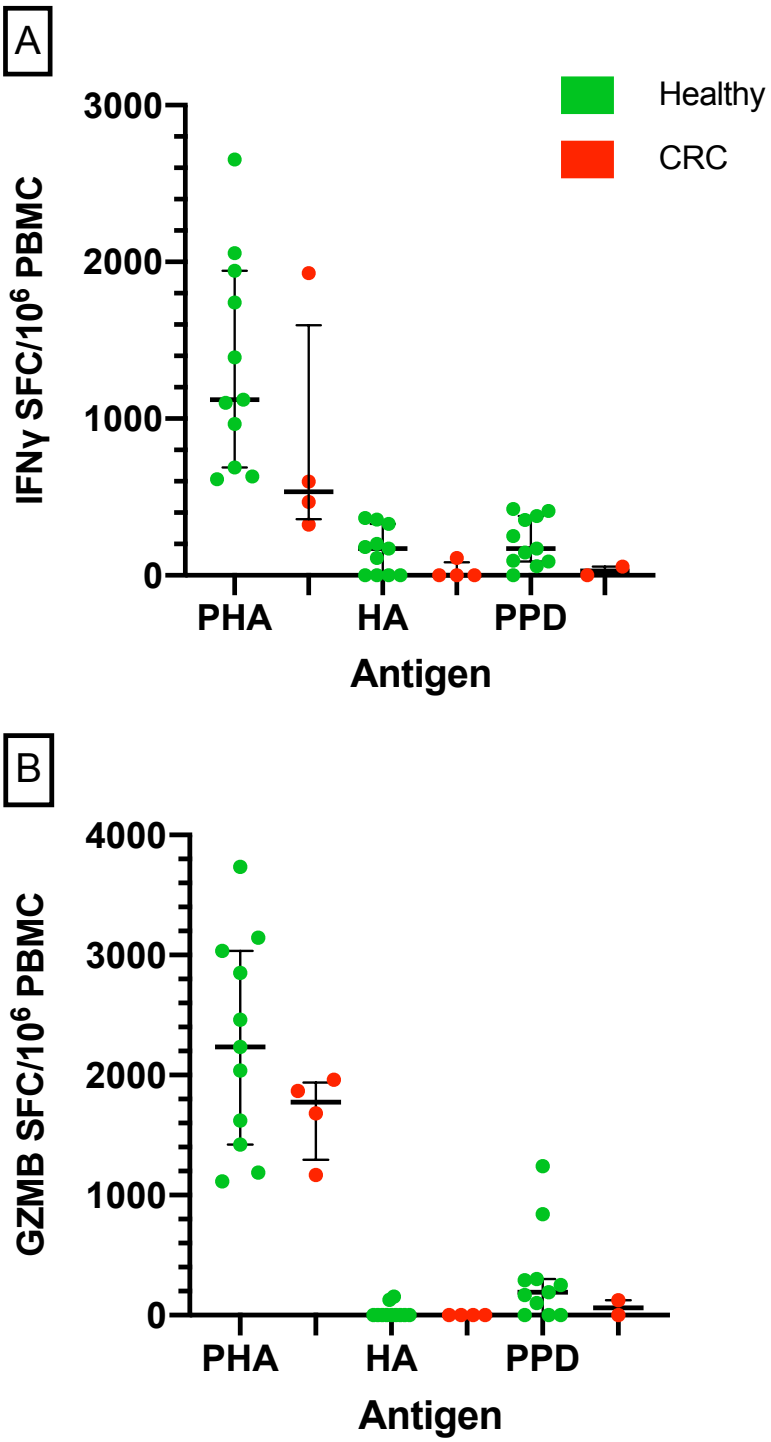


Figure 5.7 (A-B) Comparison of *ex vivo* IFNγ and GZMB T cell responses to control antigens between healthy donors and CRC patients.

Ex vivo IFNγ (A) and GZMB (B) T cell responses to control antigens PHA, HA, and PPD were measured in freshly isolated PBMC from 11 healthy donors (green) and 3-4 CRC patients (red). 2.5 x10^5 PBMC were plated per well. Magnitude of response to each antigen, normalised to spot forming cells per 10^6 PBMC, is shown for each individual. Background responses have been subtracted. Median and interquartile range shown.

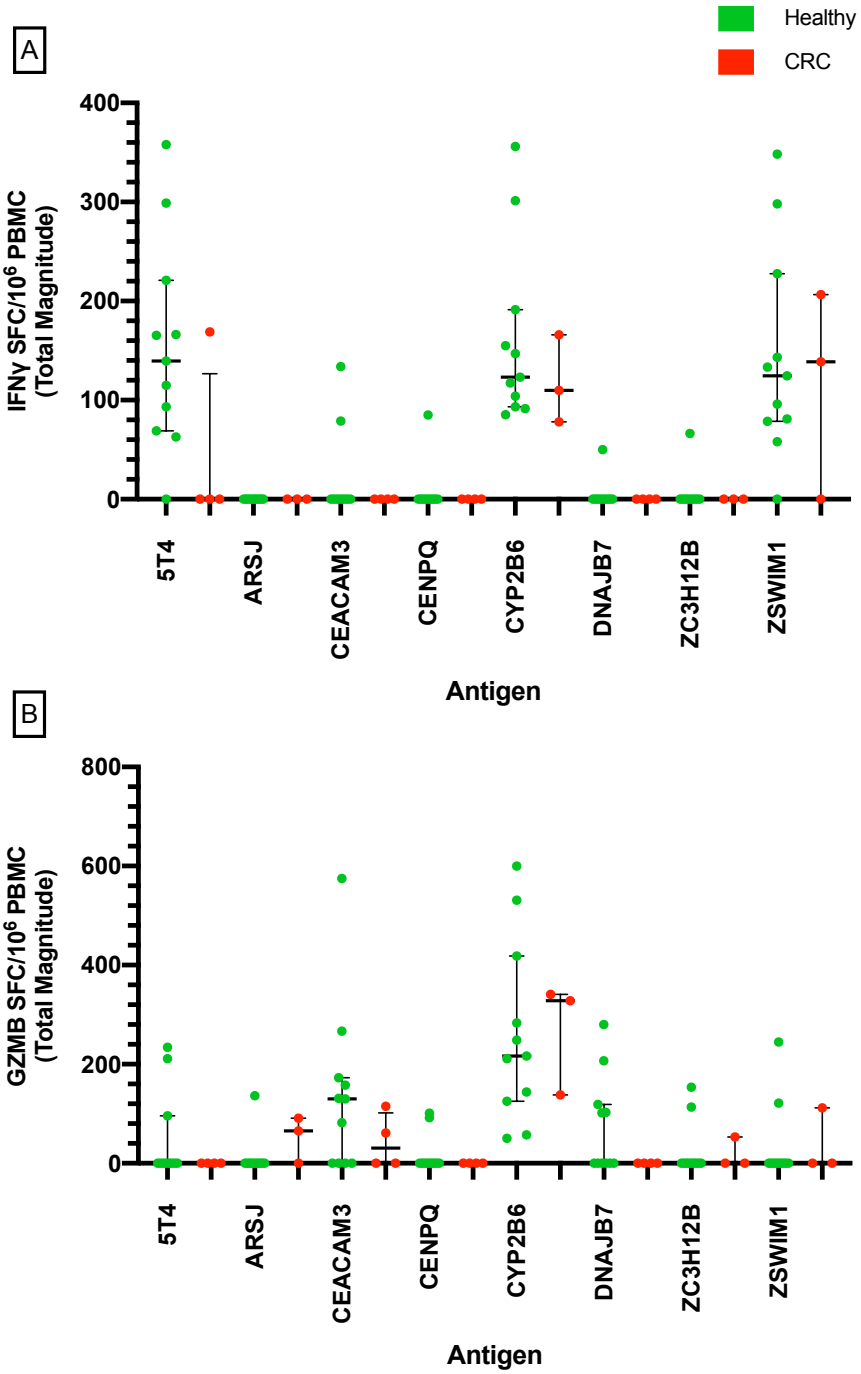


Figure 5.8 (A-B) Comparison of *ex vivo* IFNγ and GZMB T cell responses to tumour antigen 5T4 and novel tumour antigen candidates between healthy donors and CRC patients.

Ex vivo IFNγ (A) and GZMB (B) T cell responses to CRC tumour antigen 5T4, and the 7 identified candidate tumour antigens were measured in freshly isolated PBMC from 11 healthy donors (green) and 3-4 CRC patients (red). 2.5 x10^5 PBMC were plated per well. Magnitude of response to each antigen, normalised to spot forming cells per 10^6 PBMC, is shown for each individual. Background responses have been subtracted. Median and interquartile range shown.

5.2.4 Low-Frequency T Cell Responses Are Readily Identified by T Cell Expansion in Culture

The results described in Sections 5.2.1-5.2.3 have explored *ex vivo* T cell responses to control antigens, CRC tumour antigen 5T4, and novel CRC tumour antigen candidates in healthy donors and CRC patients. In general, responses identified were low frequency, therefore difficult to fully characterise. In order to further characterise the identified *ex vivo* T cell responses, short-term T cell cultures were established from healthy donor and CRC patient PBMC. Previously, the Godkin/Gallimore group have validated this approach for identifying 5T4 T cell responses, which were low-frequency or undetectable *ex vivo* in healthy donors (Clarke *et al*. 2006; Betts *et al*. 2012; Scurr *et al*. 2013).

Lines were established by stimulating PBMC with antigen peptide pools, and culturing for 14 days, as detailed in Methods. They were then restimulated, and T cell responses were investigated by IFNγ/GZMB FluoroSpot. Paired *ex vivo* and cultured experiments were performed in 4 healthy donors and 4 CRC patients, in PBMC isolated at the same time. IFNγ T cell responses were frequently immeasurable *ex vivo*, however increased responses were seen post-culture. This is shown for CRC antigen 5T4, and novel CRC antigen candidate DNAJB7 (Figure 5.9), however was also the case for the other novel antigen candidates. These findings provided clear rationale for adopting this methodology for future analysis.

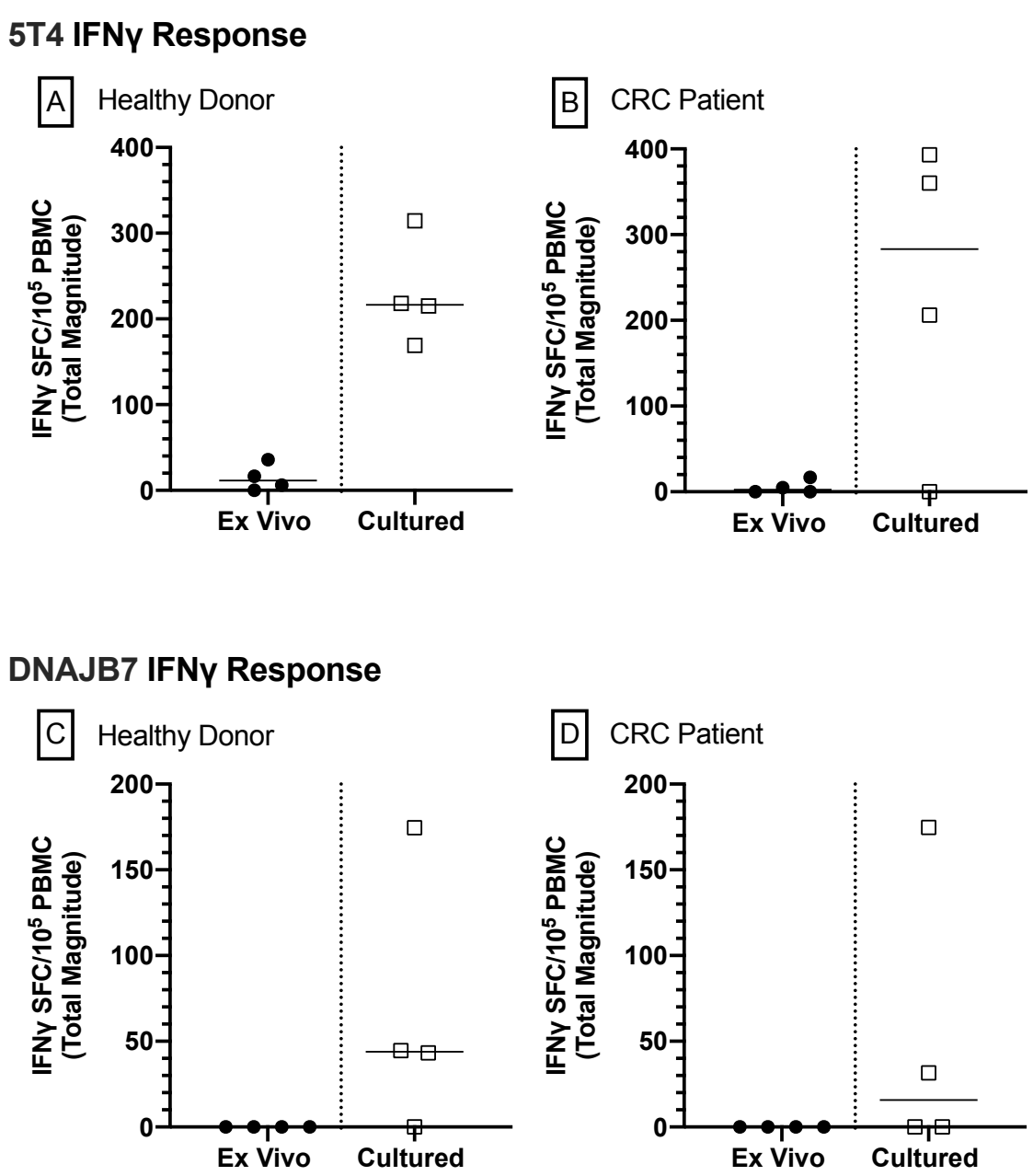


Figure 5.9 (A-D) Comparison of *ex vivo* and cultured IFNγ T cell responses to 5T4 and DNAJB7 in healthy donors and CRC patients.

Total magnitude of *ex vivo* and cultured IFNγ T cell response was compared for healthy donors (4) and CRC patients (4) to antigen 5T4 (A&B) and DNAJB7 (C&D). Total magnitude of response to each antigen, normalised to spot forming cells per 10^5 PBMC, is shown for each individual. Group median shown.

5.2.5 Cultured T Cell Responses to Novel Tumour and Control Antigens in Healthy Donors

Ex vivo Th1 responses to several of the novel tumour antigen candidates were low. In addition to this, it has been shown that IFNγ 5T4 T cell responses are significantly increased after 14-day culture and are a result of $CD45RO^+$ memory T cell responses (Scurr *et al*. 2013). This approach was validated for several of the novel tumour antigen candidates (Figure 5.9). It was hypothesised that $CD45RO^+$T cells would be responsive to the novel tumour antigen candidates; therefore, responses were assessed in 9 healthy donors using duplicate short-term cultured T cell lines. T cell responses were assessed by IFNγ and GZMB FluoroSpot, and overall magnitude of response was calculated as previously described. For each of the cultured FluoroSpot experiments shown, background activation has been subtracted. This is the number of spots seen when plating PBMC without antigen stimulation, and the background activation seen in each T cell line when they are not restimulated. Both have been subtracted from responses shown and vary between individuals. Cultured GZMB T cell responses showed, in general, a lot of background activation, making clear positive responses difficult to identify across many donors. Positive responses were only identified in a small percentage of individuals investigated (2-3) to some antigens. This Section will therefore concentrate on identified IFNγ T cell responses.

Cultured IFNγ T cell responses are shown in Figure 5.10. Positive responses were demonstrated to control antigen HA in 3/9 donors, and 8/9 donors showed responses to control antigen PPD. Clear and robust T cell responses were demonstrated for 5T4 in every individual, with a total magnitude of >200 SFC per 10^5 PBMC in 8/9 donors. Most donors showed varying levels of IFNγ T cell response to the novel antigen candidates, however some had no responses. Importantly, these non-responses were restricted to 4 donors; those who do not respond to one of these antigen candidates tend to show no responses to several. There was no obvious association with lack of response and age of donor.

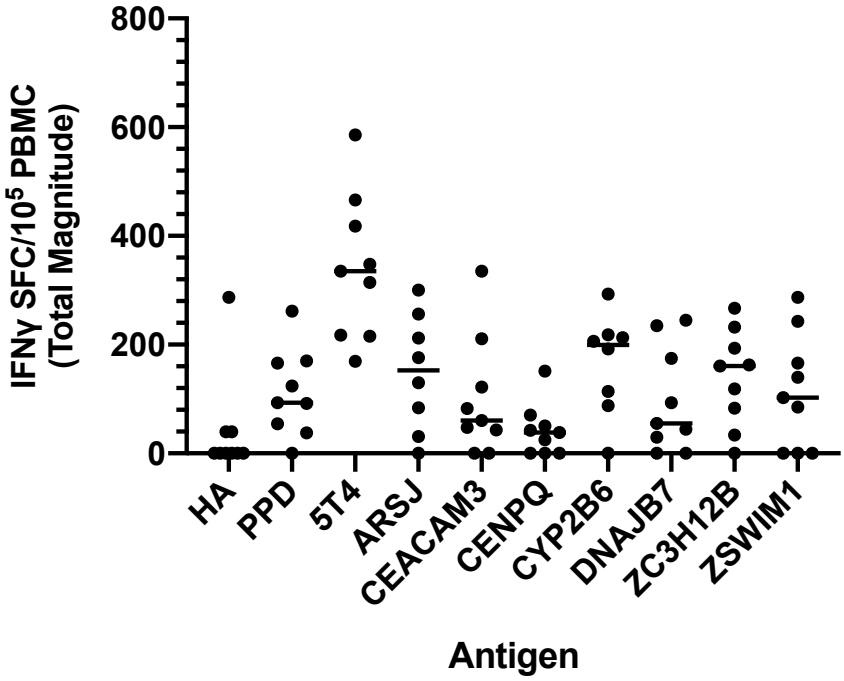


Figure 5.10 Cultured IFNγ T cell responses to control antigens, 5T4, and novel tumour antigen candidates in healthy donors.

Cultured IFNγ T cell responses to control antigens HA and PPD, known tumour antigen 5T4, and the 7 identified candidate tumour antigens were measured in short-term cultured cell lines from the PBMC of 9 healthy donors. Magnitude of response to each antigen, normalised to spot forming cells per 10^5 PBMC, is shown for each donor. Background responses have been subtracted. Group median shown.

5.2.6 Cultured T Cell Responses to Novel Tumour and Control Antigens in Colorectal Cancer Patients, and Patients with Conditions of the Colon

Short-term cultured T cell lines were also established for 8 CRC patients, and 5 patients with other conditions of the colon, to further investigate responsiveness to the novel tumour antigen candidates. This was performed as per Section 5.2.5, and similarly this Section will concentrate on the identification of IFNγ T cell responses. The patients are detailed in Materials and Methods, and are summarised below:

CRC Patients

1. Dukes' A (n=1)
2. Dukes' B (n=2)
3. Dukes' C (n=4)
4. Dukes' D (n=1)

Other Patients

1. Previous polyp adenoma, with current polyps (n=2)
2. Ulcerative colitis (UC) (n=1)
3. Recurrent benign polyps, with current polyps (n=2)

Cultured IFNγ T cell responses to control antigens HA and PPD were demonstrated in 6/7 and 8/8 CRC patients respectively (Figure 5.11A). Similarly, clear IFNγ T cell responses to 5T4 were seen in 7/8 CRC patients. With regards to the novel tumour antigen candidates, cultured IFNγ T cell responses were varied; for each antigen there were some patients who responded, and some who did not. Responses to most of the novel tumour antigen candidates, and 5T4, were generally higher in patients with benign polyps than those with current CRC or previous polyp adenoma, or with long-standing ulcerative colitis (UC) (Figure 5.11B).

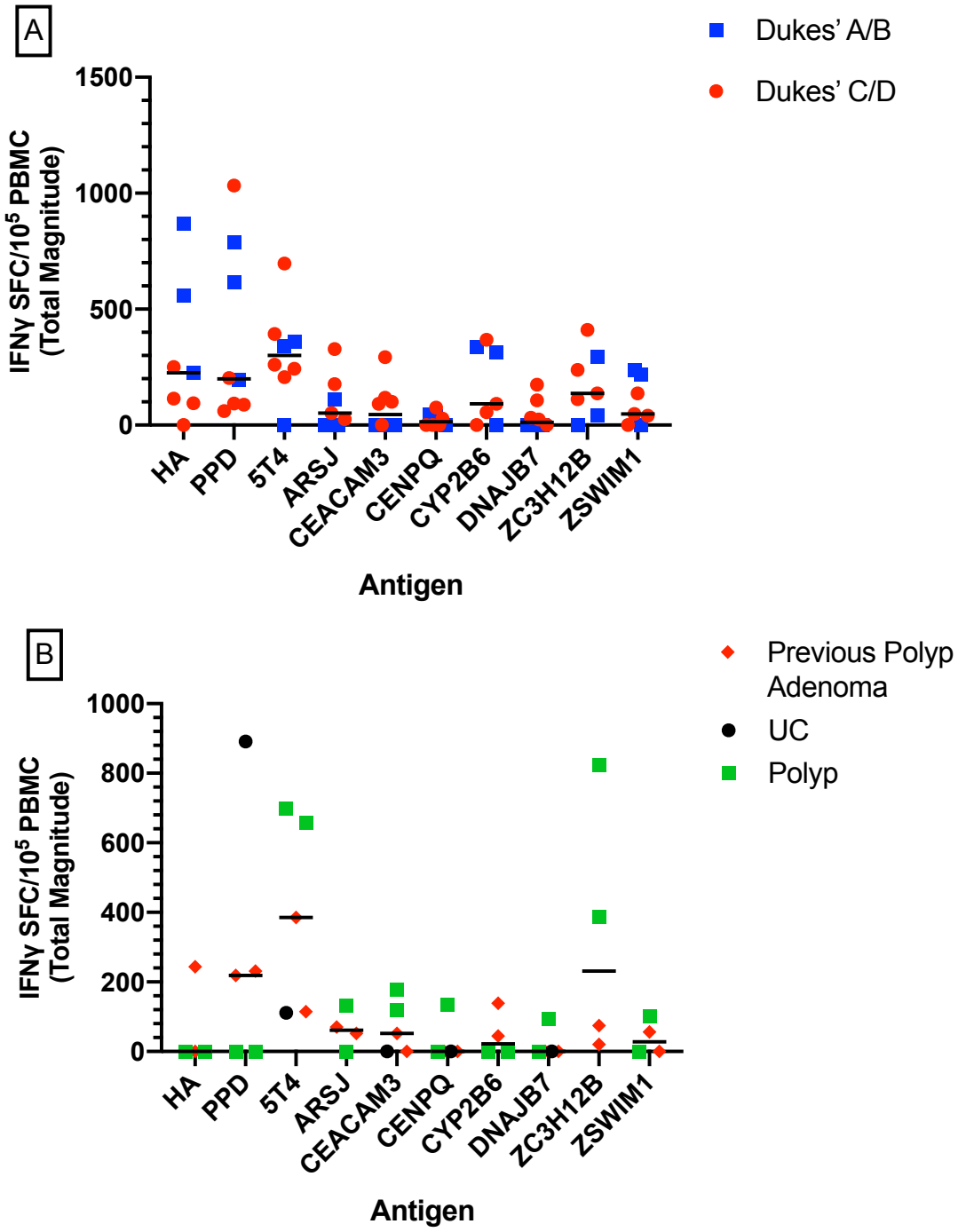


Figure 5.11 (A-B) Cultured IFNγ T cell responses to control antigens, 5T4, and novel tumour antigen candidates in CRC patients, and patients with conditions of the colon.

Cultured IFNγ T cell responses to control antigens HA and PPD, known tumour antigen 5T4, and the 7 identified candidate tumour antigens were measured in short-term cultured cell lines from the PBMC of 7-8 CRC patients (A), and 4-5 patients with conditions of the colon (B). Magnitude of response to each antigen, normalised to spot forming cells per 10^5 PBMC, is shown for each donor. Background responses have been subtracted. Group median shown.

5.2.7 Comparison of Cultured T Cell Responses to Novel Tumour and Control Antigens in Healthy Donors, CRC Patients, and Patients with Conditions of the Colon

Previous work within the Godkin/Gallimore group has identified significantly higher cultured IFNγ T cell responses to 5T4 in healthy donors compared to CRC patients. Additionally, reduced responses have also been shown to correlate with disease stage. It was therefore hypothesised that cultured IFNγ T cell responses to the identified novel tumour antigens would be altered between healthy donors and CRC patients. Cultured IFNγ T cell responses were compared for all antigens explored throughout this Chapter, and those showing differences between CRC patients and healthy donors are explored in detail in this Section.

When considering total magnitude of cultured IFNγ T cell responses to control antigens, there was no significant difference in response to PPD between heathy donors and CRC patients (Figure 5.12A). Conversely, there were significantly higher responses to HA in patients with CRC (Figure 5.12B). There was no significant difference between total magnitude of cultured IFNγ T cell response to 5T4, or the novel tumour antigen candidates, between healthy donors and CRC patients (Figure 5.13). There was, however, an indication that magnitude of response was generally lower in CRC patients than controls. This finding led to a more detailed investigation of response to 5T4, and the novel tumour antigen candidates, by considering each antigen peptide pool individually, and by stratifying patients by Dukes' stage and T stage. When considering response to each antigen across patients and healthy donors, it was also noted that individuals who lacked responses to one candidate often lacked responses to several other candidates (Table 5.1).

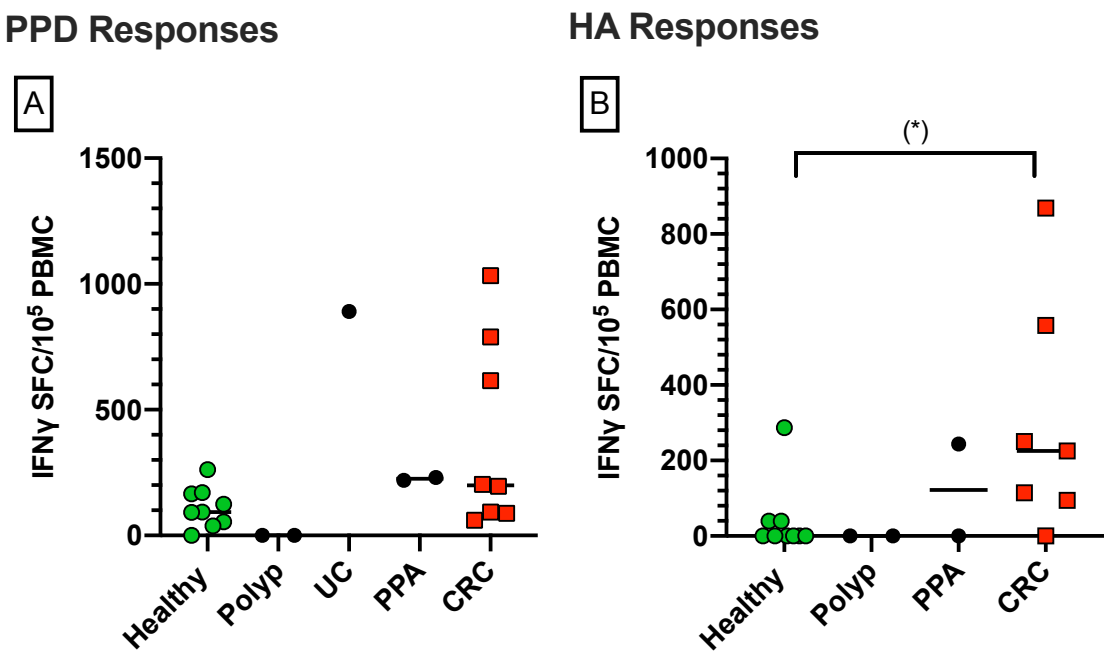


Figure 5.12 (A-B) Comparison of IFNγ cultured T cell responses to control antigens between CRC patients and healthy donors.

Cultured IFNγ T cell responses to PPD (A) and HA (B) were compared between healthy donors (9), CRC patients (7), patients with UC (0-1), and patients with previous polyp adenoma (PPA, 2). Statistical p values were calculated using Mann-Whitney U-test, as data did not follow Gaussian distribution. Indications of significance are as detailed in Methods. Group median shown.

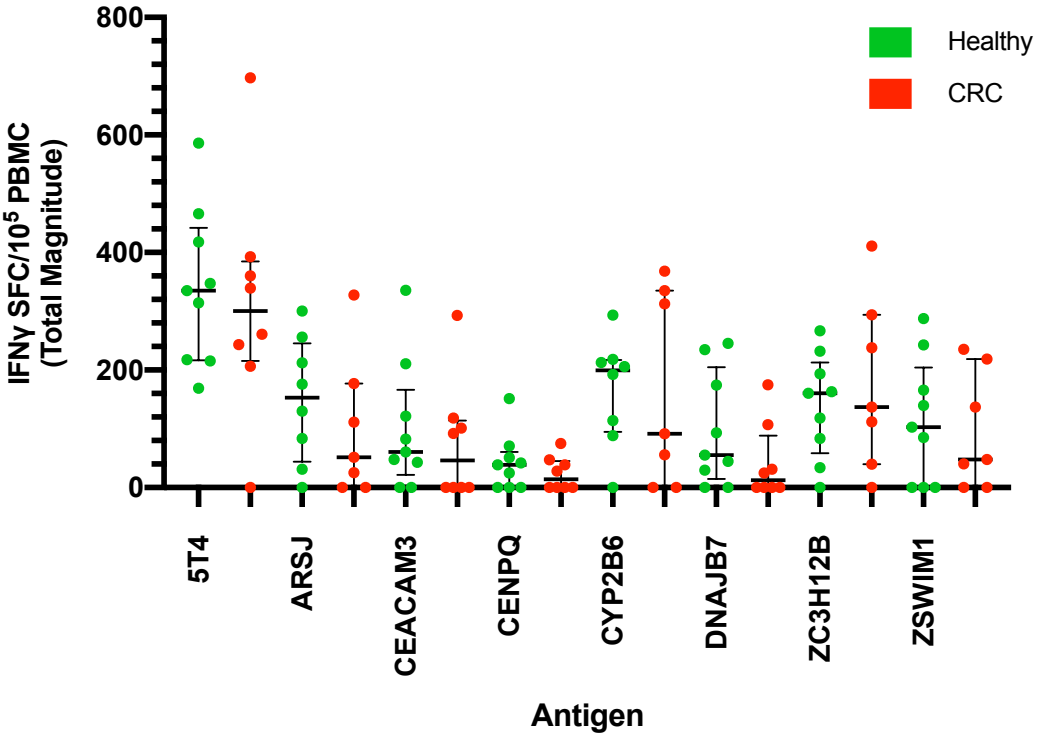


Figure 5.13 Comparison of IFNγ cultured T cell responses to 5T4, and novel tumour antigen candidates, between CRC patients and healthy donors.
Total magnitude of cultured IFNγ T cell responses to CRC tumour antigen 5T4, and each novel tumour antigen candidate were compared between healthy donors (9) and CRC patients (7). Group median shown.

Patient ID	5T4	ARSJ	CEACAM3	CENPQ	CYP2B6	DNAJB7	ZC3H12B	ZSWIM1
Healthy Donors								
CRW-EC-9	++++	n/a	+	+	n/a	-	+	-
HD1	++++	++	++++	+	+++	+	++	++
HD13	+++	+++	++	+	+++	+	++	+
HD16	++++	+	+	-	-	+	++	-
HD18	++	+	+	+	++	+	+	++
HD21	++++	++	-	-	+++	+++	++	++
HD25	++++	+++	+++	++	+++	++	+++	+++
HD28	++++	+++	+	+	+++	+++	+++	+++
HD34	+++	-	-	-	+	-	-	-

CRC Patients

CRW-D7-11	++++	++	-	+	++++	-	+++	+++
CRW-EC-1	-	-	-	-	-	-	-	-
CRW-EC-2	+++	++	+++	-	+	++	++	-
CRW-EC-3	++++	-	-	+	++++	-	+	+++
CRW-EC-4	++++	+	++	-	+	+	++	+
CRW-EC-7	+++	++++	+	+	++++	++	++++	++
CRW-EC-10	++++	+	++	+	-	+	+++	+
CRW-EC-11	+++	n/a	-	-	n/a	-	n/a	n/a

Table 5.1 Overview of cultured IFNγ T cell responses to 5T4 and the novel tumour antigen candidates for individual donors.
Cultured IFNγ T cell responses to 5T4 and the novel tumour antigen candidates for healthy donors and CRC patients (as shown in Figures 5.10 and 5.11A) are shown on a donor-by-donor basis. Magnitude of response is split based on SFC/10^5 PBMC as follows; no response =, <100 = "+", 100-200 = "++", 200-300 = "+++", >300 = "++++". Not applicable signifies that response was not tested.

Comparison of total magnitude of cultured IFNγ T cell response to 5T4 identified no significant difference between CRC patients and healthy donors (Figures 5.13 and 5.14A). When responses to each 5T4 peptide pool, PP1 and PP2, were considered individually, responses to PP1 were markedly reduced in CRC patients compared to healthy controls (Figure 5.14B). This reduced response significantly associated with increased T stage (Figure 5.14D).

Total magnitude of cultured IFNγ T cell response to CEACAM3 appeared generally lower in current/previous CRC patients and patients with UC, than in healthy donors and patients with benign polyps (Figures 5.13 and 5.15A). There was no significant difference in response to each individual peptide pool between CRC patients and healthy donors (Figure 5.15B). There was, however, a loss of response to PP1 in CRC patients with Dukes' A/B disease, compared to healthy donors, or those with Dukes' C/D disease (Figure 5.15C).

There was an overall reduction in magnitude of cultured IFNγ T cell response to DNAJB7 in CRC patients compared to healthy donors (Figures 5.13 and 5.16A). When response to each DNAJB7 peptide pool, PP1 and PP2, was considered individually, there was a significant reduction in responsiveness to PP1 in CRC patients compared to healthy donors (Figure 5.16B). Reduced responses to PP1 appear to relate to Dukes' stage and T stage (Figure 5.16C and D).

Total magnitude of cultured IFNγ T cell response to ZSWIM1 was also generally lower in CRC patients than healthy donors (Figures 5.13 and 5.17A). This reduced response was clearly demonstrated when each peptide pool was considered individually; response to PP3 was markedly reduced in CRC patients compared to controls (Figure 5.17B). The loss of response to PP3 appeared to associate with increased T stage (Figure 5.17D).

Total magnitude of cultured IFNγ T cell response to ARSJ, CENPQ, CYP2B6, and ZC3H12B were not significantly different between healthy donors and CRC patients (Figure 5.13). When each peptide pool was considered alone, there were still no significant differences between groups (Figure 5.18 A-D). Similarly, no differences were demonstrated when CRC patients were stratified by either Dukes' stage, or T stage (data not shown).

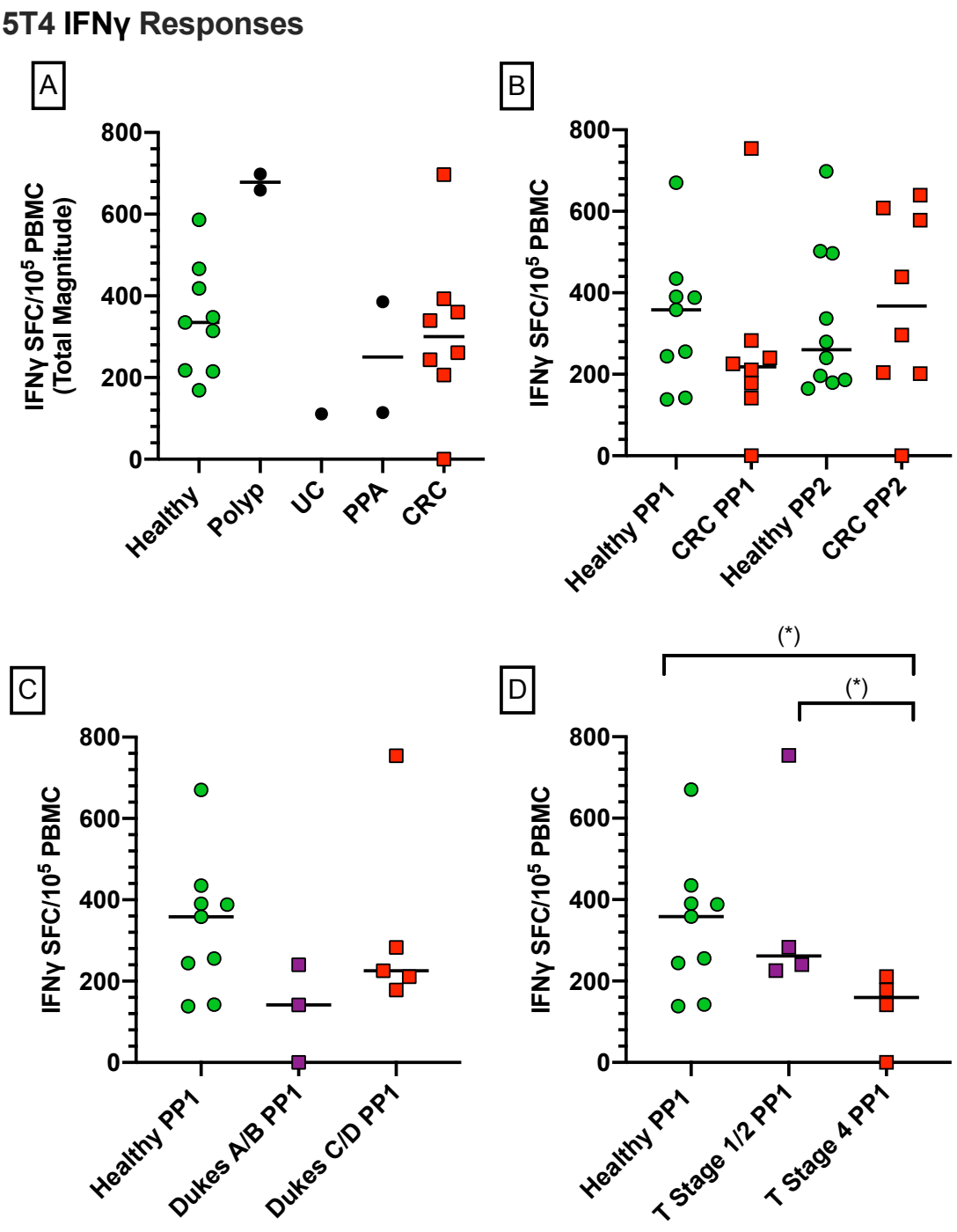


Figure 5.14 (A-D) Cultured IFNγ T cell responses to 5T4 are significantly lower in CRC patients and healthy donors.

Total magnitude of cultured IFNγ T cell response to tumour antigen 5T4 was compared between healthy donors (9), CRC patients (8), benign polyp patients (2), UC patients (1), and patients with previous polyp adenoma (PPA, 2) (A). Average response to each peptide pool, normalized to SFC/10^5 PBMC, was compared between healthy donors and CRC patients (B). Average response to PP1 was also compared between healthy donors and CRC patients stratified by Duke's stage (C), and T stage (D). Statistical p values were calculated using Mann-Whitney U-test. Indications of significance are as detailed in Methods. Group median shown.

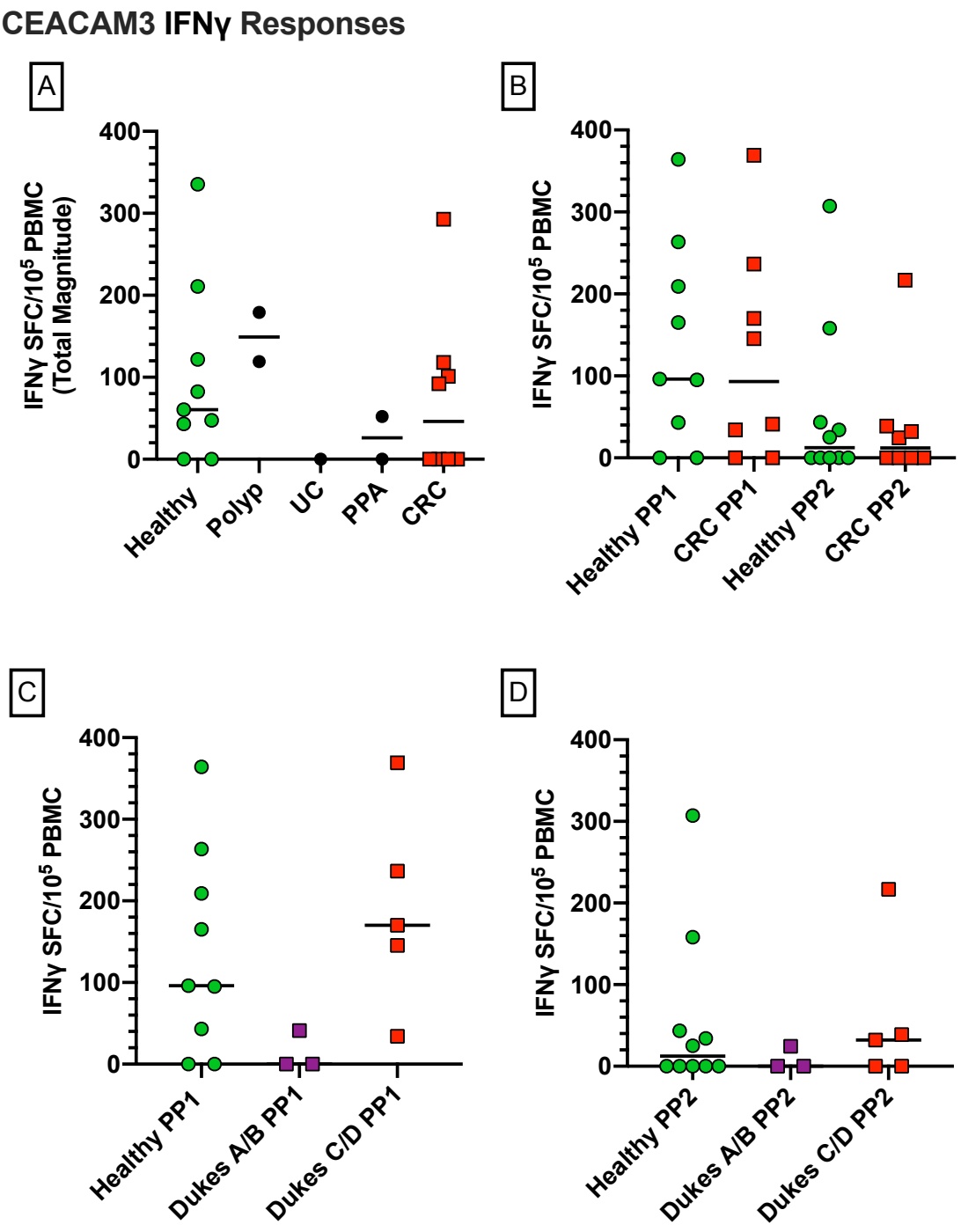


Figure 5.15 (A-D) Cultured IFNγ T cell responses to CEACAM3 are lower in CRC patients than healthy donors.

Total magnitude of cultured IFNγ T cell response to novel tumour antigen candidate CEACAM3 was compared between healthy donors (9), CRC patients (8), benign polyp patients (2), UC patients (1), and patients with previous polyp adenoma (PPA, 2) (A). Average response to each peptide pool (PP1 and PP2), normalized to SFC/10^5 PBMC, was compared between healthy donors and all CRC patients (B), or CRC patients stratified by Dukes' stage for each peptide pool individually (C and D). Group median shown.

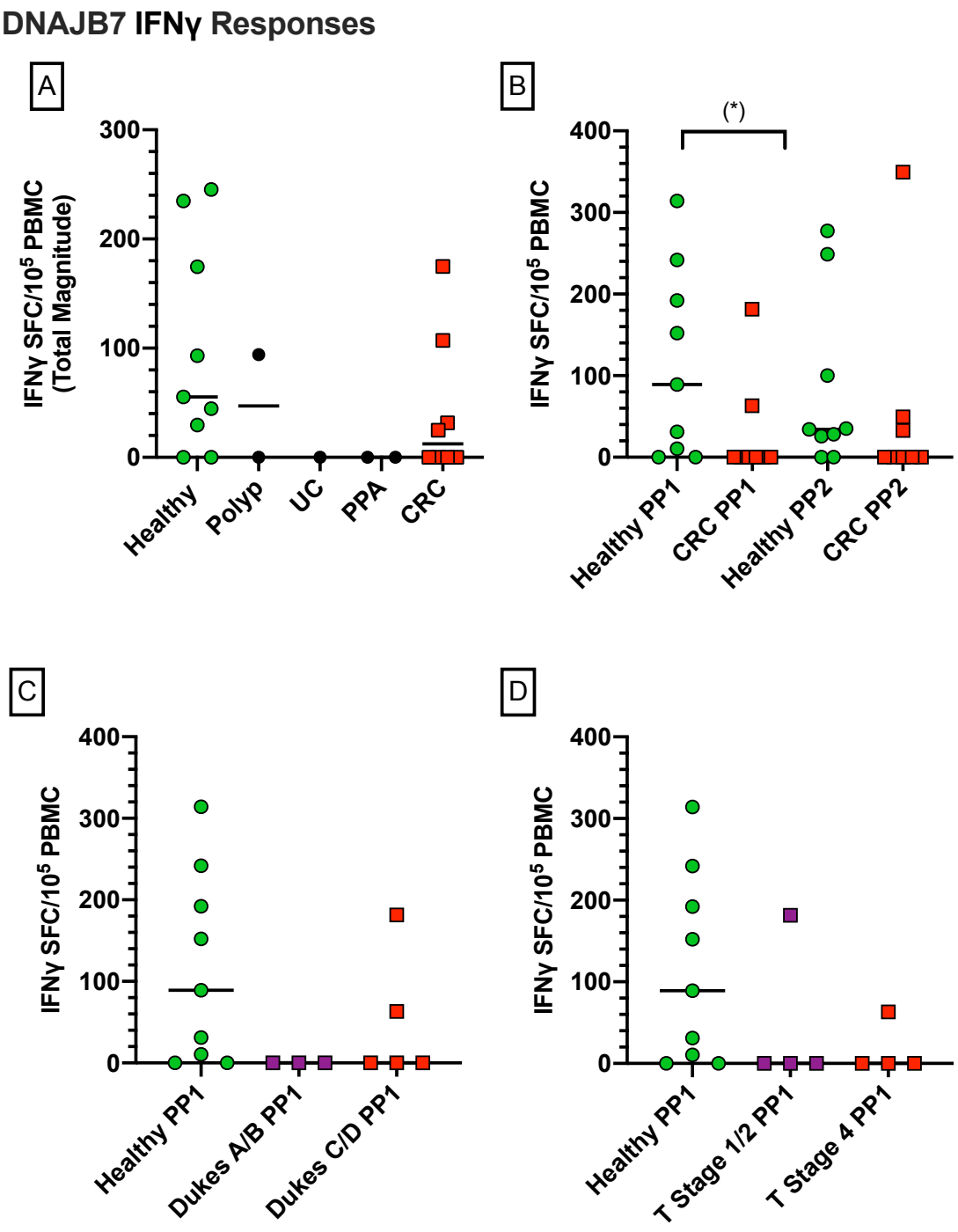


Figure 5.16 (A-D) Cultured IFNγ T cell responses to DNAJB7 are significantly lower in CRC patients than healthy donors.

Total magnitude of cultured IFNγ T cell response to novel candidate antigen DNAJB7 was compared between healthy donors (9), CRC patients (8), benign polyp patients (2), UC patients (1), and patients with previous CRC (2) (A). Average cultured IFNγ T cell response to each peptide pool, normalized to SFC/10^5 PBMC, was compared between all healthy donors and all CRC patients (B). Average cultured IFNγ T cell response to PP1 was also compared between healthy donors, and CRC patients stratified by either Dukes' stage (C), or T stage (D). Group median shown.

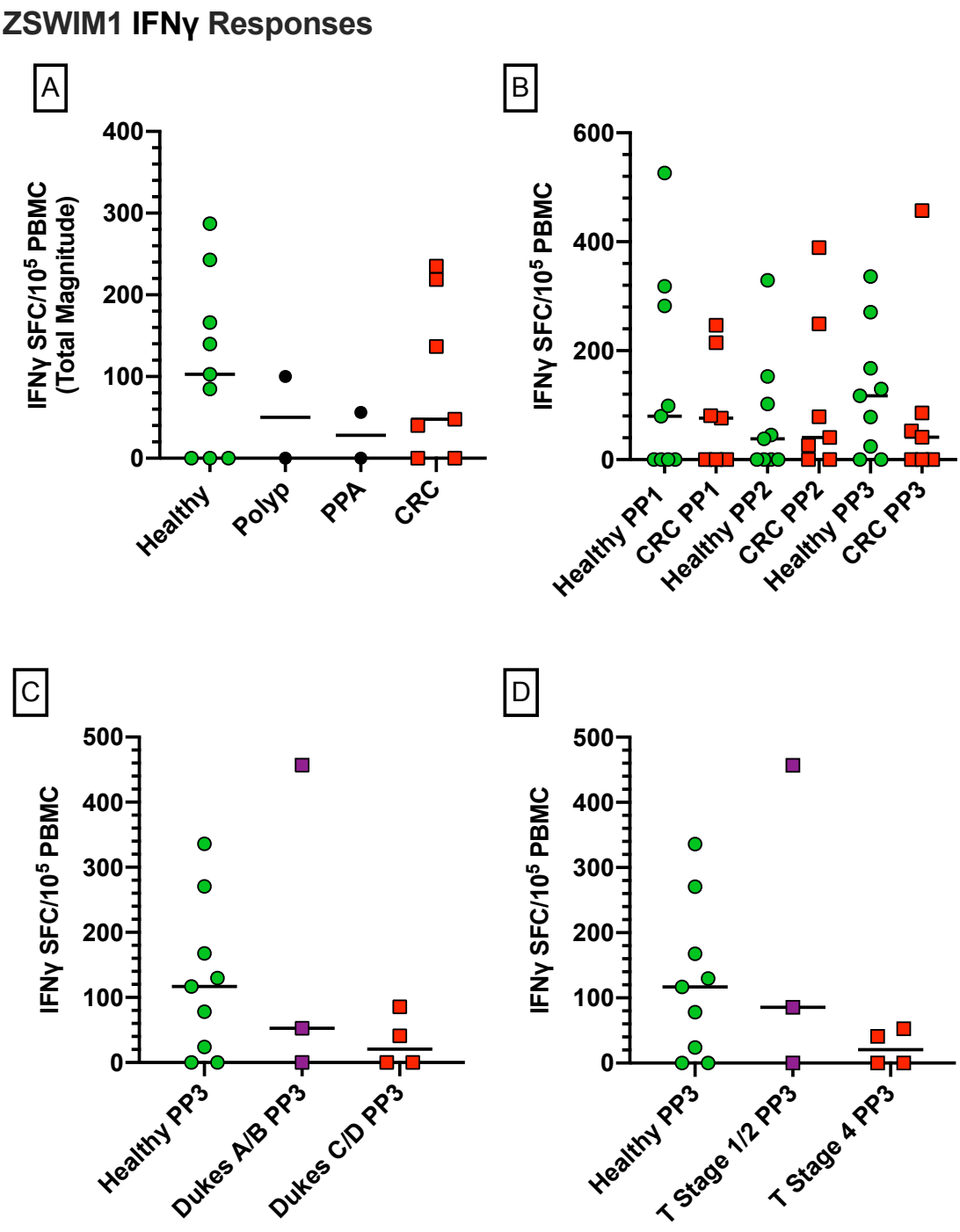


Figure 5.17 (A-D) Cultured IFNγ T cell responses to ZSWIM1 are significantly lower in CRC patients than healthy donors.

Total magnitude of cultured IFNγ T cell response to novel candidate antigen DNAJB7 was compared between healthy donors (9), CRC patients (8), benign polyp patients (2), and patients with previous polyp adenoma (PPA, 2) (A). Average cultured IFNγ T cell response to each peptide pool, normalized to SFC/10^5 PBMC, was compared between all healthy donors and all CRC patients (B). Average cultured IFNγ T cell response to PP3 was also compared between healthy donors, and CRC patients stratified by either Dukes' stage (C), or T stage (D). Group median shown.

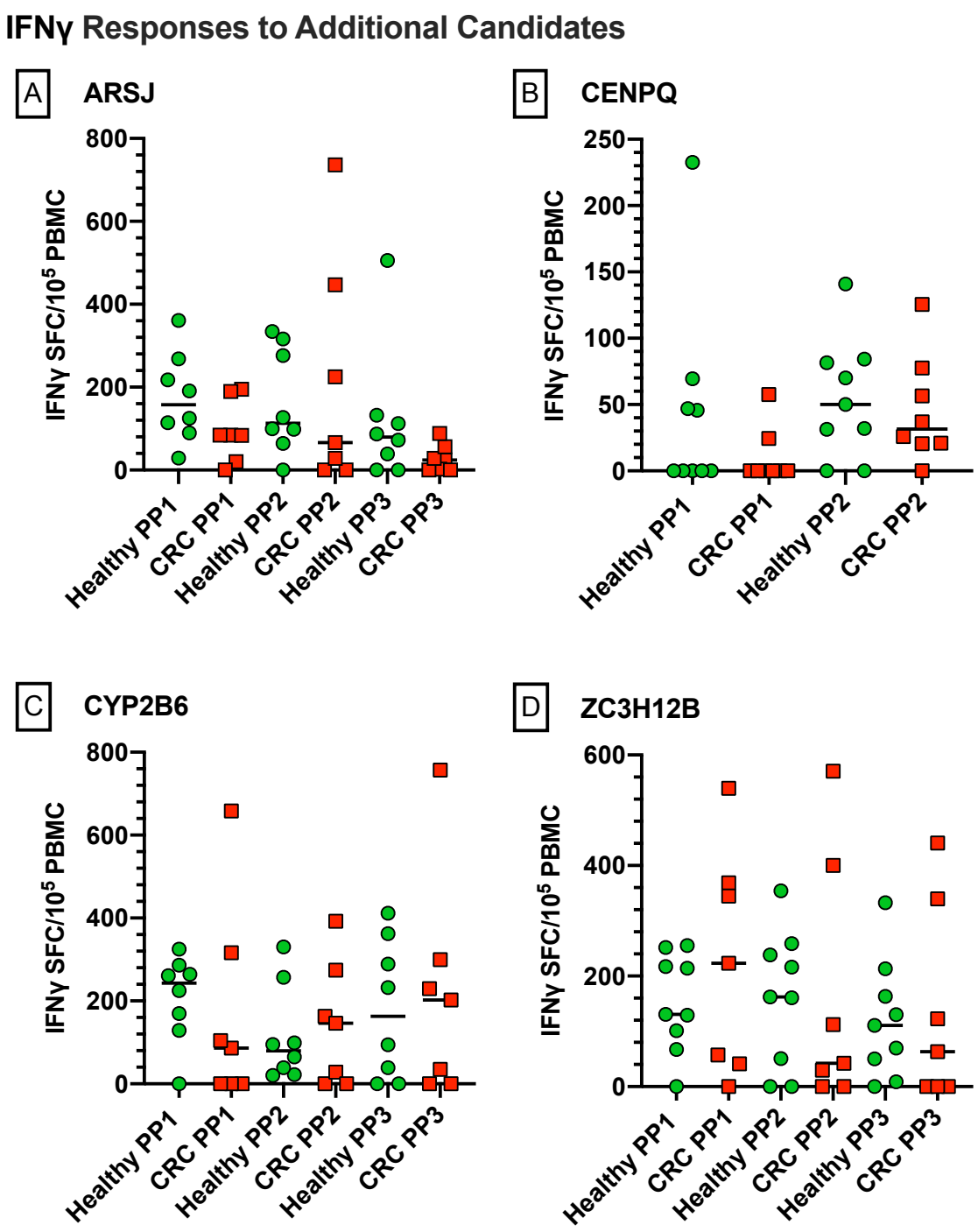


Figure 5.18 (A-D) Comparison of cultured IFNγ T cell responses to ARSJ, CENPQ, CYP2B6, and ZC3H12B between healthy donors and CRC patients. Average cultured IFNγ T cell response to each individual peptide pool of novel antigen candidates ARSJ (A), CENPQ (B), CYP2B6 (C), ZC3H12B (D) was compared between healthy donors (8-9) and CRC patients (7-8). Group median shown.

5.2.8 Effect of Cyclophosphamide Treatment on T Cell Responses to Novel Tumour Antigens in Advanced Metastatic CRC Patients from the TaCTiCC Clinical Trial

Thus far, the results described in this Chapter have confirmed the immunogenicity of several novel CRC tumour antigen candidates. Moreover, patterns of IFNγ T cell response to some of these candidates, namely CEACAM3, DNAJB7, and ZSWIM1, were reduced in patients with CRC compared healthy controls. This was most pronounced in patients with advanced disease. TaCTiCC demonstrated that treatment of advanced mCRC patients with CPM increased IFNγ T cell responses to 5T4, particularly in those patients who responded to therapy. It was therefore hypothesised that T cell responses to the identified antigens would also be increased during CPM treatment, in CPM responders. This hypothesis was investigated using frozen PBMC of 5 advanced mCRC patients receiving CPM treatment, collected during the TaCTiCC clinical trial. 14-day cultures were established and stimulated with the novel tumour antigen candidates as previously described. Th1 and Th2 T cell responses were assessed using IFNγ/IL-4/IL-10 tricolour FluoroSpot assays.

The number of live cells thawed from each frozen PBMC sample was counted using fluorescence-activated cell sorting (FACS) with live/dead marker Propidium Iodide (PI). Given that these samples had been frozen for 2-4 years, these data, in addition to known number of cells at the time of freezing, were used to calculate percentage cell recovery. Cell recovery was generally low, frequently falling below 50% (Figure 5.19). When considering percentage recovery in relation to period of CPM treatment, there was some indication that recovery rate was further reduced during periods of CPM treatment (Figures 5.19 and 5.20). Cell numbers were, therefore, far lower than anticipated, meaning subsequent analysis was limited.

The low percentage cell recovery may result in reduced cell viability in culture, therefore the methodology for assessing cultured antigen T cell responses was adapted accordingly. After 14 days in culture, live cell number of each cultured line was calculated by FACS and live/dead staining with PI. FluoroSpot assays were performed as previously described, however magnitude of response was normalised to SFC/10^5 live PBMC. It should be noted that percentage of live cells after 14 days in culture was highly variable between lines, and between patients.

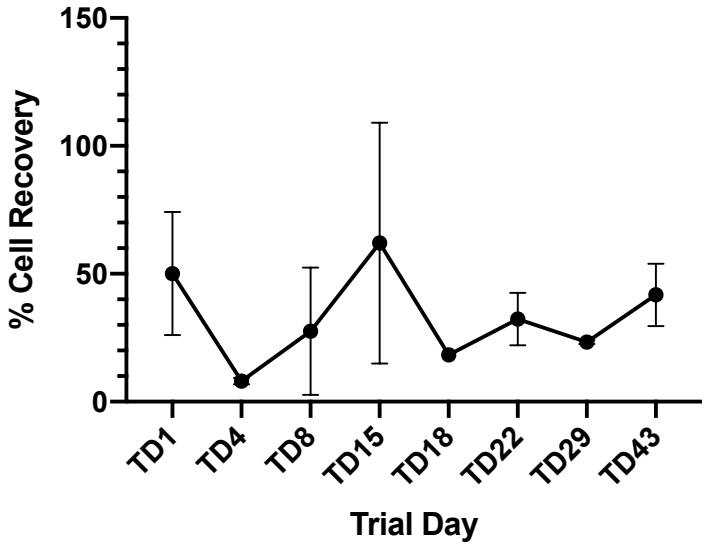


Figure 5.19 Percentage cell recovery of frozen TaCTiCC PBMC samples.
Live cell number thawed from frozen PBMC sample was counted by FACS, using PI as a live/dead marker, in 6 TaCTiCC patients receiving CPM treatment. Limited numbers of cells were frozen; therefore, a different number of patients were assessed at each timepoint; TD1 = 5, TD4 = 2, TD8 = 2, TD15 = 5, TD18 = 1, TD22 = 5, TD29 = 2, TD43 = 3. Group mean plus SD shown.

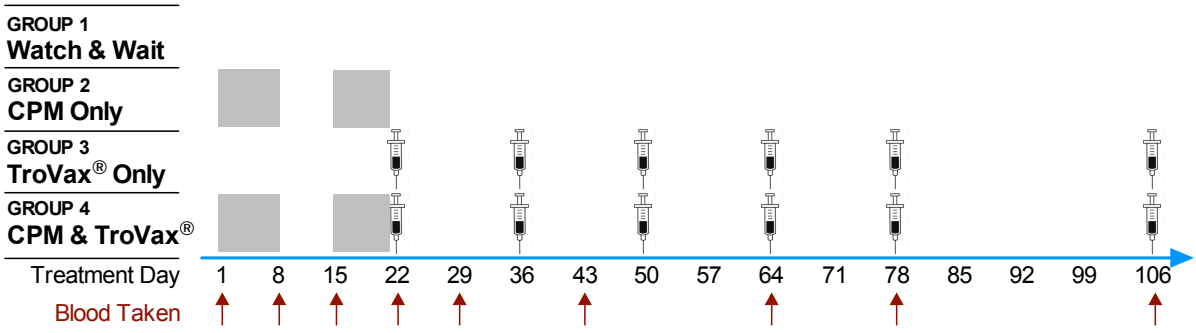


Figure 5.20 TaCTiCC treatment schedule.
TaCTiCC treatment schedule (Adapted from (Scurr *et al.* 2017a). In applicable groups, CPM treatment was given in 2 blocks; between TD1 and TD8, then between TD15 and TD22, and TroVax injections were subsequently given as indicated.

The cultured IFNγ T cell response to 5T4 was assessed throughout the TaCTiCC trial. Given the low level of cell recovery, it was a concern that frozen responses may not mirror those seen in fresh PBMC. Cultured IFNγ 5T4 T cell responses using frozen PBMC were therefore investigated across CPM-treatment, in 2 CPM-responder TaCTiCC patients. These responses were assessed against the responses identified in fresh PBMC of the same donor during TaCTiCC. Importantly, total magnitude of IFNγ 5T4 T cell response during TaCTiCC was identified using 13 5T4 peptides, whereas those for the frozen PBMC were identified using 2 larger 5T4 peptide pools. Additionally, 5T4 T cell responses in the frozen samples were normalised to live cell number, whereas those during TaCTiCC were not. These data are therefore not directly comparable. Cultured IFNγ T cell response to 5T4 was, however, clearly increased during CPM treatment in both patients, using fresh and frozen PBMC (Figure 5.21).

Cultured IFNγ/IL-4/IL-10 T cell responses to control antigen PPD, and novel tumour antigen candidates CEACAM3, DNAJB7, and ZSWIM1 were investigated in the frozen PBMC of 5 TaCTiCC patients; 101, 102, 109, 113, and 116. All of these patients are known CPM responders, with the exception of patient 116. T cell responses to 5T4 were investigated in patients 101 and 102.

Treatment with CPM led to increased magnitude of cultured IFNγ T cell response to CEACAM3 and DNJAB7 in some patients, however there was no clear response pattern (Figure 5.22). Increased cultured IFNγ T cell responses to ZSWIM1 were found during CPM treatment in 4/4 patients assessed for ZSWIM1 responses. There was no clear pattern of change in cultured IL-4 or IL-10 T cell response to 5T4, or the novel tumour antigen candidates, upon treatment with CPM (Figures 5.23 and 5.24).

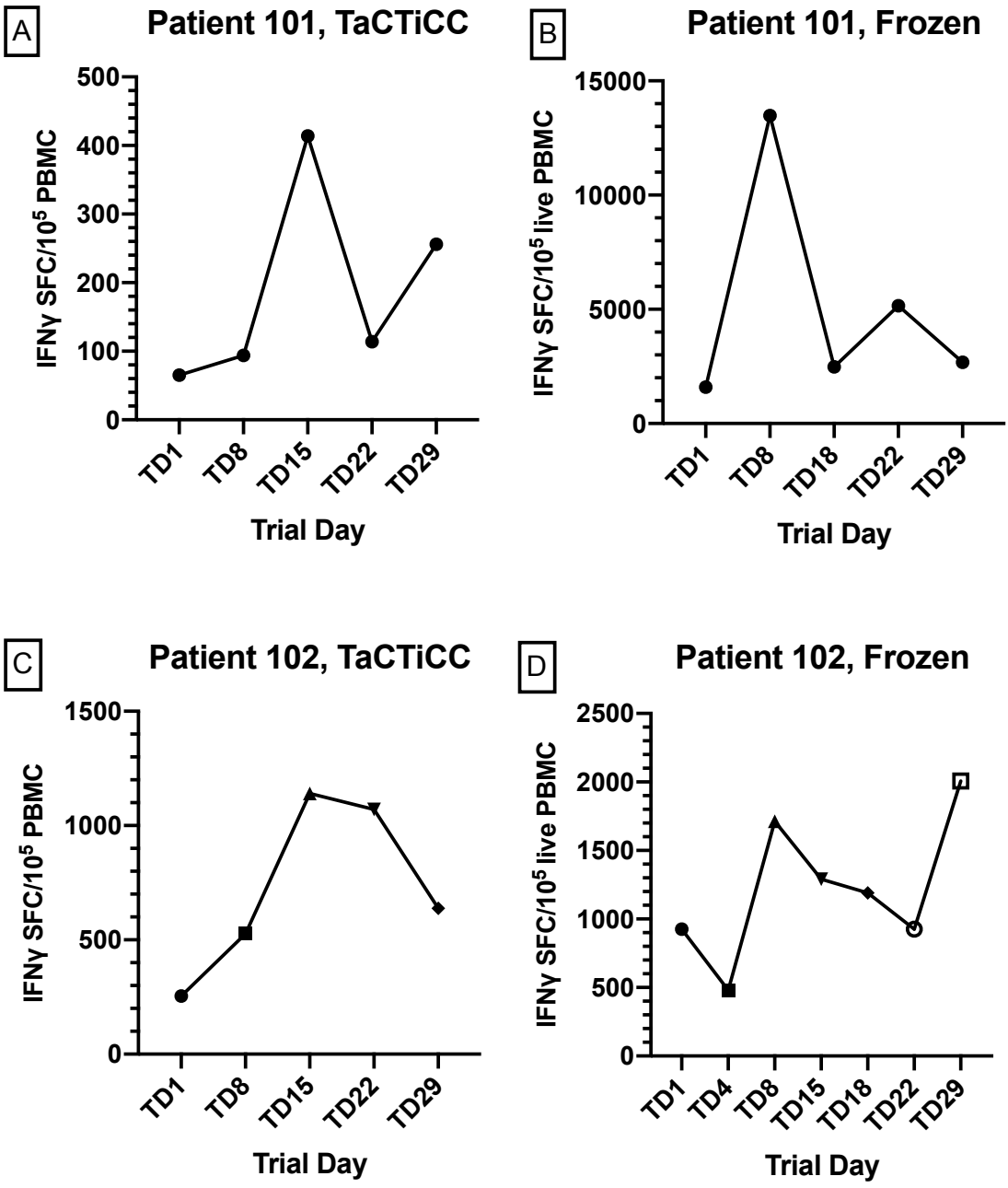


Figure 5.21 (A-D) Comparison of cultured IFNγ 5T4 T cell responses from fresh/frozen PBMC in TaCTiCC patients receiving CPM treatment.

Cultured IFNγ 5T4 T cell response from fresh PBMC as identified during TaCTiCC in two patients receiving CPM-treatment, normalised to SFC/10^5 PBMC (A and C). IFNγ 5T4 T cell response were investigated using frozen PBMC from the same patients, normalised to SFC/10^5 live cells post-culture (B and D). Total magnitude of response shown for each timepoint, and background responses have been subtracted.

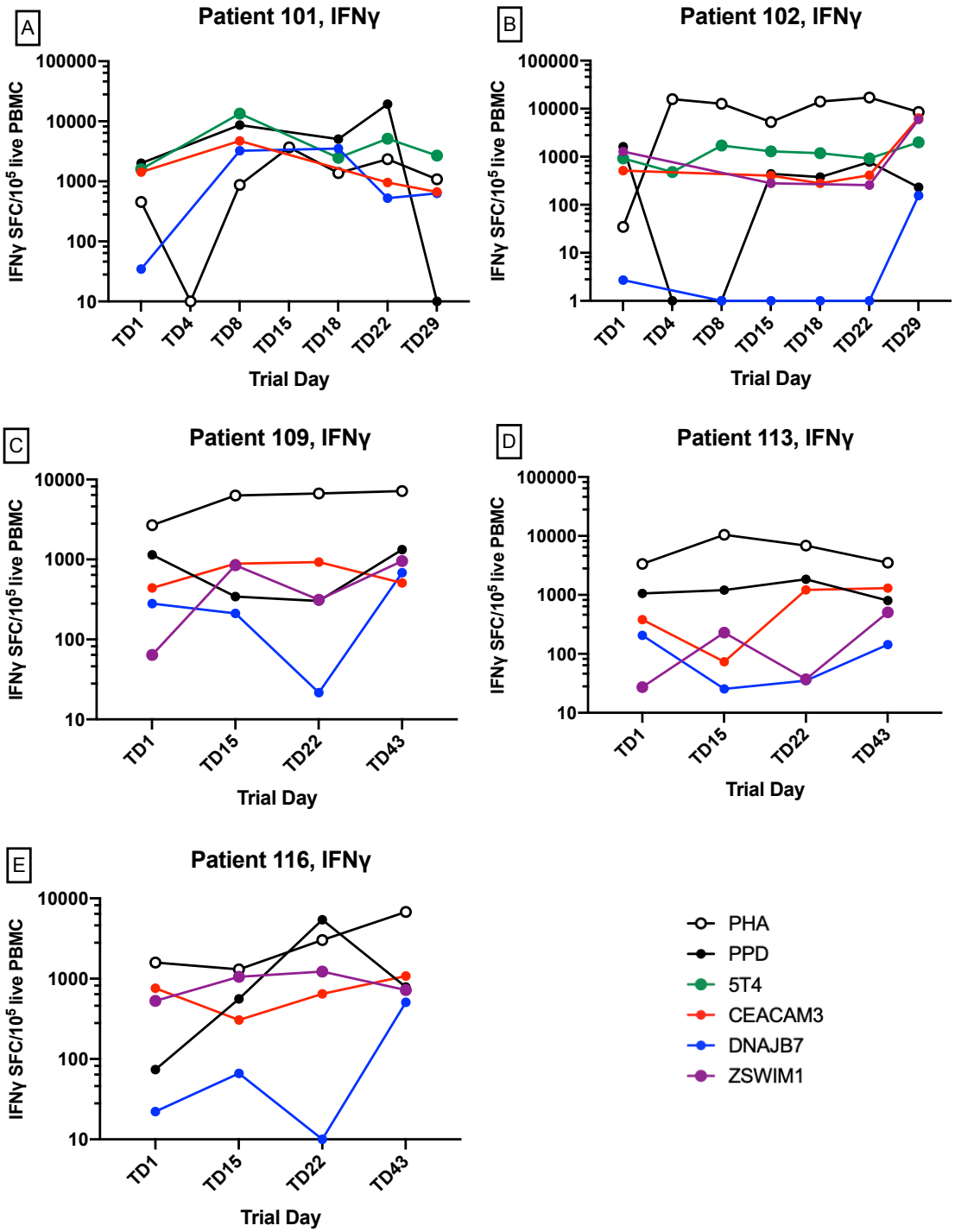


Figure 5.22 (A-E) Cultured IFNγ T cell responses from frozen TaCTiCC PBMC in patients receiving CPM treatment.

Total magnitude of cultured IFNγ T cell response to PPD, 5T4, CEACAM3, DNAJB7, and ZSWIM1 was investigated in the frozen PBMC of 5 TaCTiCC patients during CPM treatment (A-E). Response to each peptide pool was normalised to live cell number and used to calculate total magnitude of response to each antigen. Background responses have been subtracted.

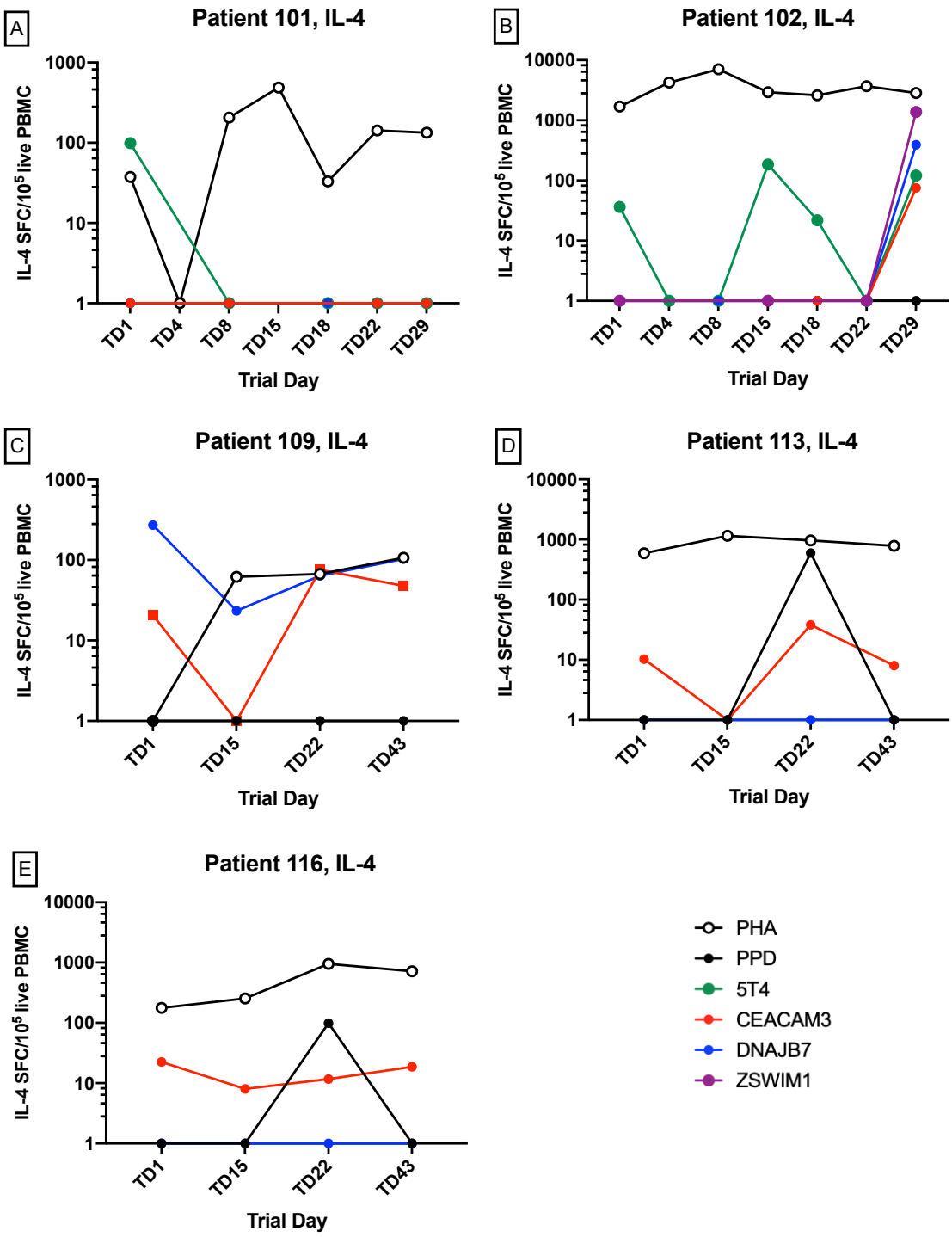


Figure 5.23 (A-E) Cultured IL-4 T cell responses from frozen TaCTiCC PBMC in patients receiving CPM treatment.

Total magnitude of cultured IL-4 T cell response to PPD, 5T4, CEACAM3, DNAJB7, and ZSWIM1 was investigated in the frozen PBMC of 5 TaCTiCC patients during CPM treatment (A-E). Response to each peptide pool was normalised to live cell number and used to calculate total magnitude of response to each antigen. Background responses have been subtracted.

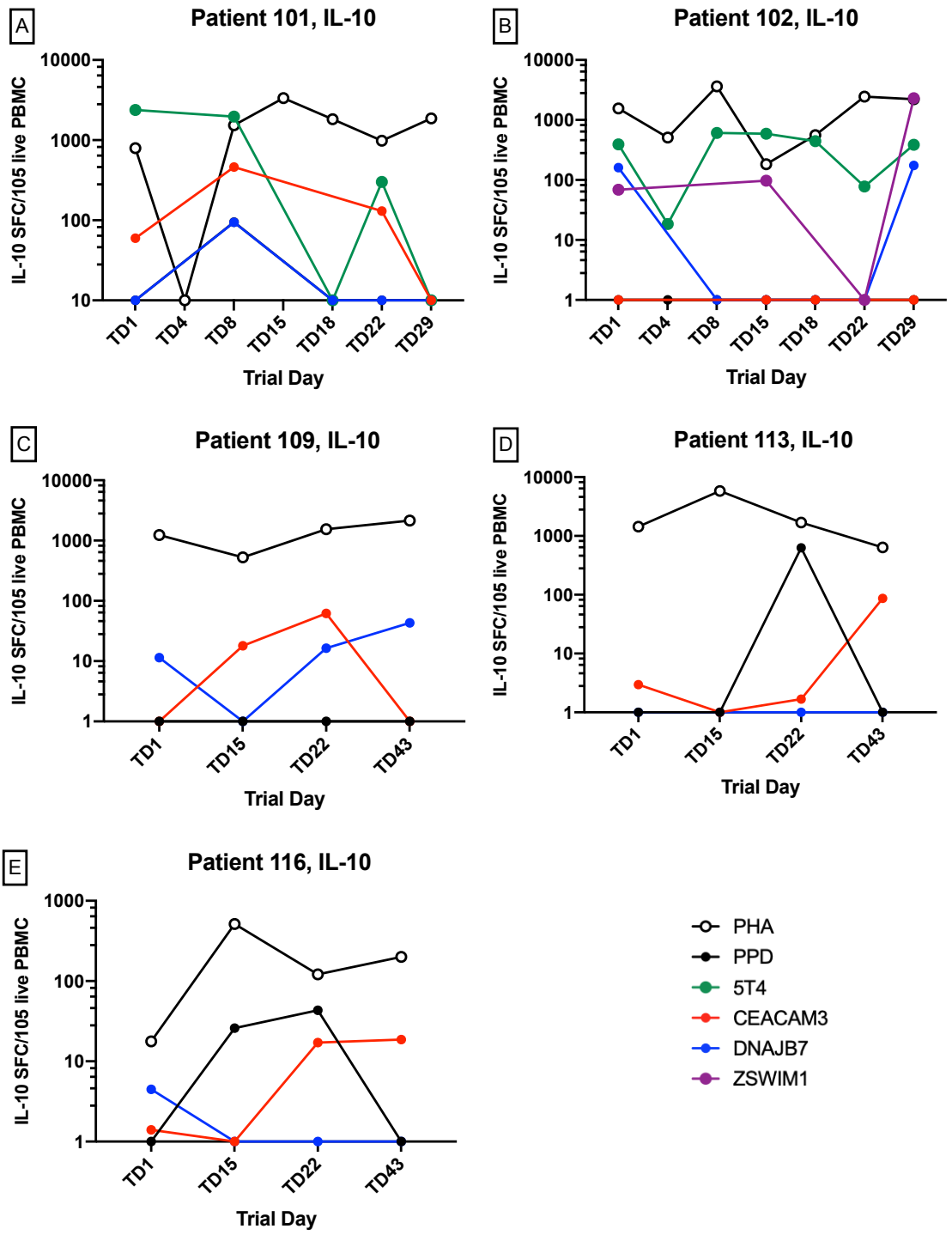


Figure 5.24 (A-E) Cultured IL-10 T cell responses from frozen TaCTiCC PBMC in patients receiving CPM treatment.

Total magnitude of cultured IL-10 T cell response to PPD, 5T4, CEACAM3, DNAJB7, and ZSWIM1 was investigated in the frozen PBMC of 5 TaCTiCC patients during CPM treatment (A-E). Response to each peptide pool was normalised to live cell number and used to calculate total magnitude of response to each antigen. Background responses have been subtracted.

5.2.9 DNAJB7; Novel Tumour Antigen Target for Cancer Immunotherapy

The results discussed in this Chapter have confirmed the immunogenicity of several novel CRC antigen candidates. One such candidate is DNJAB7, Th1 T cell responses to which are significantly reduced in CRC patients compared to controls. It was hypothesised that DNABJ7 therefore represents a potential target for CRC immunotherapy. To further explore the viability of DNAJB7 as a target, publicly available data on protein and RNA expression from the Human Protein Atlas was assessed (Uhlén *et al*. 2015). Each of the novel tumour antigens discussed in this Chapter had increased expression in CRC tissue compared to healthy tissue, with the exception of ARSJ. DNAJB7, however, exhibited the most optimal expression profile, with lack of RNA and protein expression in healthy tissues, with the exception of testis and placenta. Interestingly, DNAJB7 expression was also found in other tumours, for example breast and lung. DNAJB7 may, therefore, represent a novel cancer-testis antigen. This expression data, together with the described finding that IFNγ T cells responses to DNAJB7 are reduced in CRC patients, identified DNAJB7 as an attractive target for CRC immunotherapy.

In-house immunohistochemistry (IHC) to assess protein expression of DNAJB7 was optimised in order to confirm the results of the Human Protein Atlas, and to allow for future investigation. Staining was comparable in healthy testis and colon tissue, and in CRC tumours (Figure 5.25). Importantly, low-level cytoplasmic expression was demonstrated in the healthy colon epithelium. This expression was, however, not as high as that seen in healthy testis tissue, or CRC tumours.

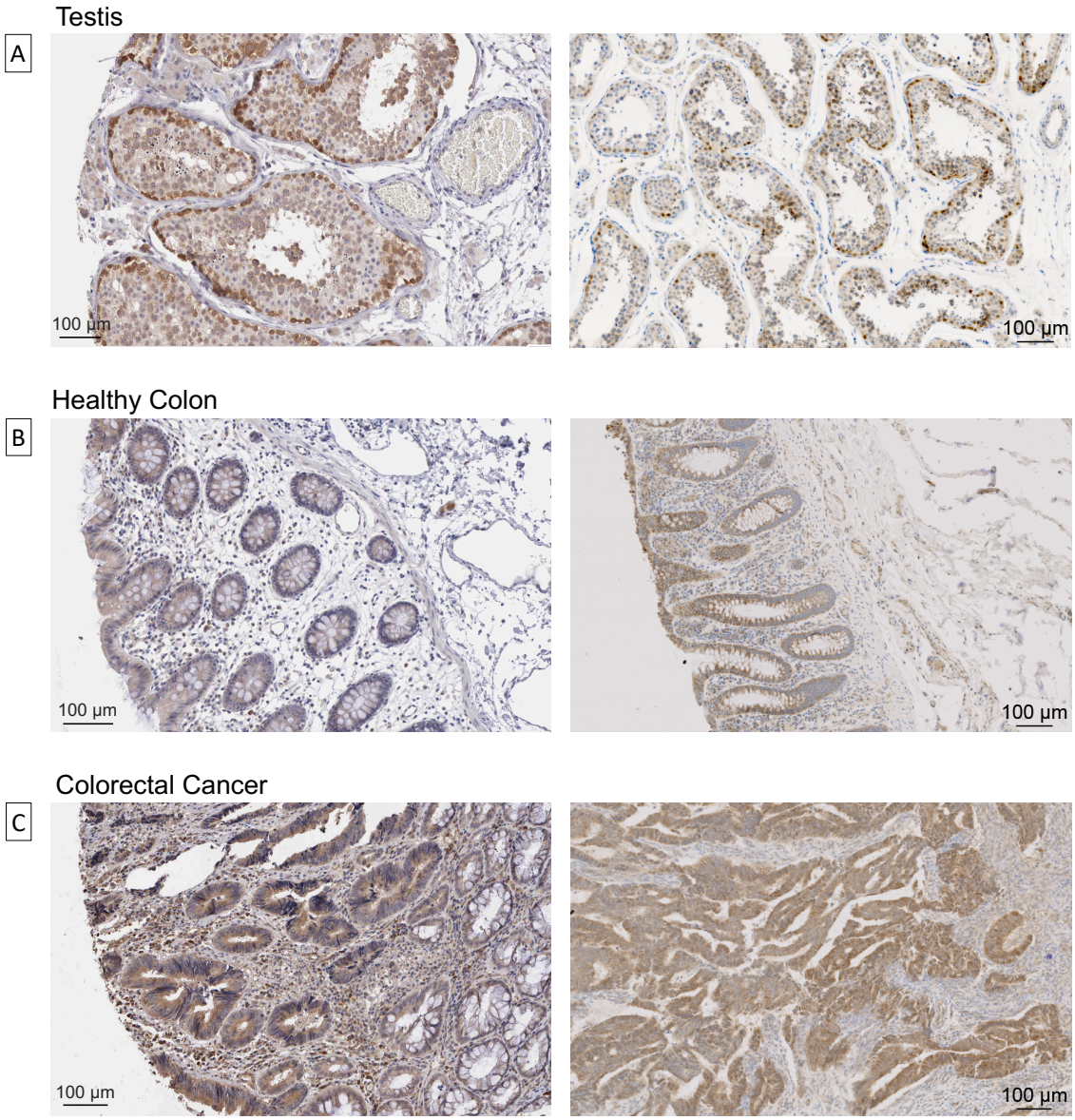


Figure 5.25 (A-C) Immunohistochemical staining of DNAJB7 in testis tissue, healthy colon, and CRC.

DNAJB7 staining was optimised (right-hand images) and compared to that available on the Human Protein Atlas (left-hand images). Comparable expression was found in healthy testis (A&B), healthy colon (C&D), and CRC tissue (E&F). High expression in testis tissue appears confined to the seminiferous ducts (A&B), whereas low-level cytoplasmic expression is demonstrated in epithelial cells of healthy colon (C&D). High cytoplasmic expression was demonstrated in CRC tumour cells (E&F).

5.3 Discussion

Using RNA sequencing, the Godkin/Gallimore group identified 7 novel candidate tumour antigens which were upregulated in purified epithelial cells from CRC tumours compared to healthy background tissue; ARSJ, CEACAM3, CENPQ, CYP2B6, DNAJB7, ZSWIM1, and ZC3H12B. In the study described in this Chapter the immunogenicity of these candidates was explored in healthy donors, CRC patients, and patients with conditions of the colon, using pools of 20mer peptides overlapping by 10 amino acids, covering the entire sequence of each protein. Subsequent ImmunoSpot analysis was used to quantify immune responses to these peptides, both *ex vivo* and in short-term cultured T cell lines. It is likely that the identified responses to several of these candidates are the result of antigen recognition by $CD45RO^{+}$ memory T cells. The effect of CPM treatment on responses to some of these antigens was also investigated in frozen PBMC samples from advanced mCRC patients recruited to the TaCTiCC clinical trial. Consideration of these findings, and publicly available protein and RNA expression data from the Human Protein Atlas, identified DNAJB7 as a novel tumour-testis antigen, and potential target for CRC Immunotherapy.

Robust Antigen-Specific T Cell Responses in Healthy Donors

Each of the identified candidate antigens represents a protein with a range of normal physiological functions. It was therefore somewhat surprising to find such robust immune responses mounted against these proteins in healthy donors, raising important questions surrounding the existence and maintenance of such T cell responses. During T cell development, thymic selection utilizes positive and negative selection to retain only those T cells able to correctly function, and to eliminate those recognizing self-peptide. In addition to this, peripheral tolerance mechanisms exist to ensure the control of self-reactive T cells which have escaped central tolerance. Failure to control self-reactive T cells can lead to a range of autoimmune diseases.

There is increasing evidence supporting the existence of circulating T cells recognising normal self-proteins in both cancer patients, and healthy controls (Campi *et al*. 2003; Danke *et al*. 2004; Clarke *et al*. 2006; Scurr *et al*. 2013; Scurr *et al*. 2015). Similarly, robust T cell responses have been shown against tumour-antigens, for example 5T4. It is hypothesised that these responses may be involved in immunosurveillance of the colon. It is possible that these immune responses may reflect ongoing tumour immunosurveillance. This is a reasonable hypothesis, considering that

many of the identified candidate tumour antigens are involved in cell signalling pathways which are aberrant in cancerous cells.

Decreased T Cell Responses to CEACAM3, DNAJB7, and ZSWIM1 in CRC Patients Compared to Healthy Donors

Even in the small number of donors investigated, this Chapter has shown that cultured Th1 responses to CEACAM3, DNAJB7, and ZSWIM1 are reduced in CRC patients compared to healthy donors. These observations are similar to those seen for CRC tumour antigen 5T4. Increased immunosuppression is well-described in cancer (Wolf *et al*. 2003; Diaz-Montero *et al*. 2009), and is the basis for many immunotherapeutic strategies. In addition, it is known that Th1 responses are instrumental in the development of anti-tumour immune responses (Hung *et al*. 1998). The significant reduction in responses to the identified candidate tumour antigens may, therefore, reflect loss of Th1 immunity associated with cancer development. Furthermore, it is possible that loss of these responses is accompanied by increased immune-regulation and suppression. The significant reduction in Th1 immune responses to these proteins in CRC patients supports that their normal physiological role may be directly or indirectly anti-tumorigenic.

DNAJB7 is a member of the heat shock protein 40kD (Hsp40) family. These proteins are known molecular chaperones involved ensuring correct protein folding and preventing protein aggregation, which become upregulated during periods of stress. Accordingly, the aberrant expression of these proteins has been described in cancer (Isomoto *et al*. 2003; Kanazawa *et al*. 2003). It is an attractive hypothesis that immune-mediated control mechanisms exist to eliminate cells in extreme conditions of stress.

CEACAM3, is a glycoprotein and is expressed on granulocytes. It is a member of the CD66 immunoglobulin family, however unlike other CD66 family members which are involved in cell-to-cell adhesion, CEACAM3 functions as an innate immune receptor targeting bacterial pathogens for destruction (Schmitter *et al*. 2004; Schmitter *et al*. 2007). T cell responses against CEACAM3 peptides may, therefore, represent a method of targeted immune activation and/or homing, linking innate and adaptive immunity. It is important to consider that CEACAM3 belongs to the same family as CRC tumour antigen CEA, also known as CEACAM5. T cell responses against CEA have been demonstrated in both healthy donors and CRC patients (Campi *et al*. 2003). Additionally, increased CEA-specific Th1 responses pre-resection were found to identify CRC patients with

increased risk of disease relapse (Scurr *et al*. 2015). T cells targeting CEA in CRC patients are known to induce severe side effects, including colitis (Parkhurst *et al*. 2011).

Comparatively little is known about the function of ZSWIM1. It is known to be expressed in leukocytes, particularly in naïve $CD4^+$ lymphocytes, and contains a zinc finger ZSWIM motif. It is thought to play a role in the development of T helper cells (Ko *et al*. 2014). Th1 mediated responses to ZSWIM1 may, therefore, be important in the development/function of naïve T cells.

Limitations

The work described in this Chapter is subject to several unavoidable limitations. Access to the PBMC of healthy, age-matched donors is limited therefore healthy donors described in this Chapter are much younger than the CRC patients. It cannot, therefore, be rejected that differences in T cell responses to novel tumour antigen candidates CEACAM3, DNAJB7, and ZSWIM1 are not a result of age-related immune decline. This would, however, be unlikely considering that significant differences in response were also seen within the CRC patient group, relating to tumour stage. Previous work within the lab also identified no clear link between age of CRC patient and magnitude of response to 5T4. Nonetheless, even if immune responses to CEACAM3, DNAJB7, and ZSWIM1 were reduced in an age-dependant manner, it does not necessarily invalidate their use as CRC Immunotherapy targets, as risk and incidence of disease increase with age. This work should be extended to a group of older healthy donors.

Diseased patients were recruited during their pre-operation clinic appointment; therefore, disease status was frequently uncertain. This led to the recruitment of patients with a range of non-cancerous bowel conditions, and of patients with diverse CRC staging. This has significantly impacted the number of CRC patients investigated for each stage of disease. Recruitment to scientific studies is also unavoidably limited by disease prevalence and is contingent on patient consent and participation. It would be important to extend the work described to an increased number of patients with diseases of the colon, and stage-specific CRC patients.

An additional concern when recruiting donors is PBMC count. Frequently, there were lower PBMC numbers isolated from the same volume of blood in CRC than healthy donors. This may be a result of chemotherapy and/or immunosuppressive drugs and adds additional practical limitations on the number of replicates, and experimental

conditions it is possible to investigate in one individual. This limitation is unavoidable and unpredictable.

Experiments performed using frozen TaCTiCC PBMC were critically limited the live cell number recovered from each frozen sample. Moreover, the live cell number after 14-day culture was low in many of the established cell lines in each patient. Although normalising response by live cell number allows the comparison of these results, efforts should be made to repeat this investigation in freshly isolated PBMC of patients receiving CPM treatment. In addition to these considerations, it has been shown that T cell responses to several control antigens; HA, PHA, PPD, naturally fluctuate across time in the same individual (Figure 5.3). This may be a result of altered magnitude of immune response, however, may also simply be a drawback of performing several discrete ImmunoSpot assays. This should be considered when interpreting the data shown for 5T4 collected during the TaCTiCC clinical trial. Experiments performed using frozen TaCTiCC samples were, however, performed at the same time for each individual patient.

Preliminary experiments within the Godkin/Gallimore using freshly isolated PBMC of a post-colectomy CRC patient receiving metronomic CPM suggest that DNAJB7 may follow a similar pattern to 5T4; increased responses are demonstrated during treatment, as T_{reg} numbers are depleted (Figure 5.26). Future studies are planned within the Godkin/Gallimore to investigate the therapeutic benefit of low-dose CPM in earlier-stage CRC patients. This trial presents an excellent opportunity to assess the effect of CPM on T cell responses to the newly identified candidate tumour antigens.

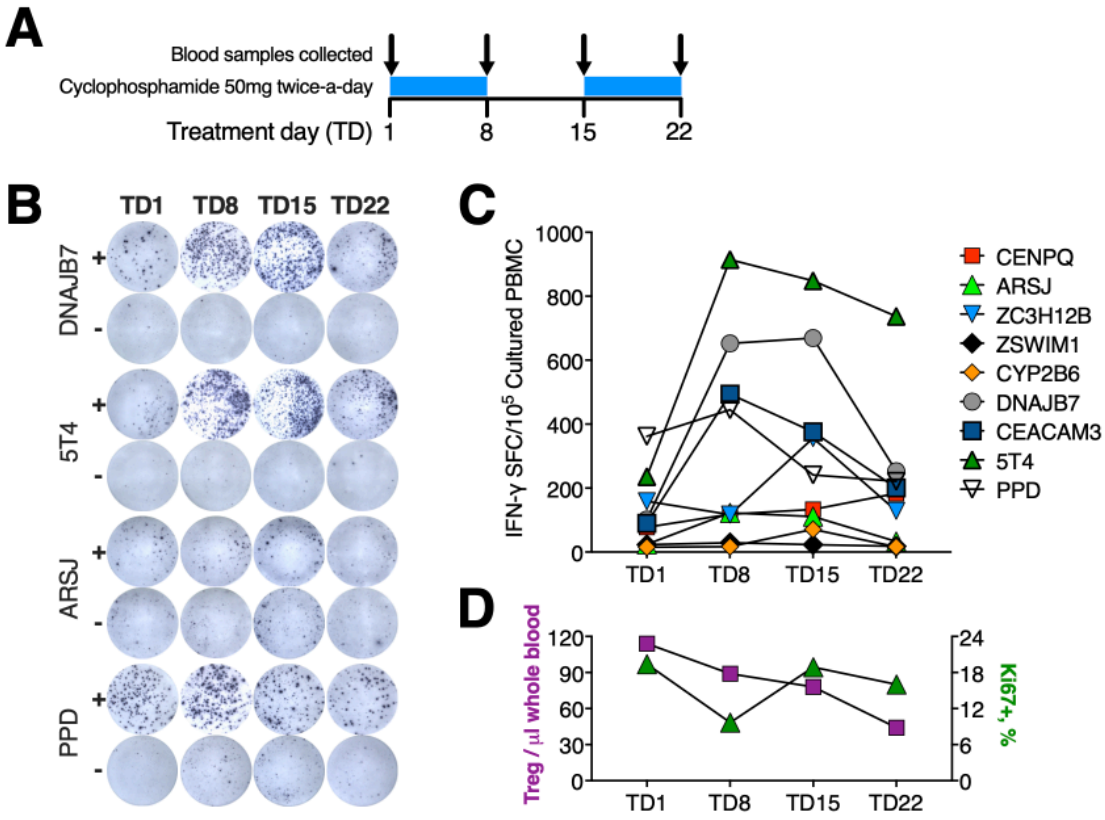


Figure 5.26 (A-D) Th1 responses to certain novel TAAs are unmasked by regulatory T cell depletion in CRC.

A post-colectomy CRC patient received low-dose, metronomic CPM on treatment days 1-8 and 15-22, with blood samples collected weekly throughout treatment (A). T cell responses to peptide pools spanning the entire protein sequence of each candidate TAA were assessed by cultured IFNγ ELISpot at each timepoint (B). The total number of IFNγ SFC/10^5 cultured PBMC were calculated for each of the novel tumour antigen candidates (C). $CD3^+CD4^+CD25^{hi}Foxp3^+$ regulatory T cell numbers and $\%Ki67^+$ T_{regs} were measured by flow cytometry during CPM treatment (D). This is unpublished work from the Godkin/Gallimore group.

Future Directions

The results discussed in this Chapter have identified potential memory Th1 responses to several novel tumour antigen candidates. Those seen for CEACAM3, DNAJB7, and ZSWIM1 are reduced in CRC patients compared to healthy donors, with clear trends associating with tumour stage. These results are in line with those described for known CRC tumour antigen 5T4. It would be interesting to confirm the T cell subsets responsible for these responses. This could be investigated by CD4/CD8/CD45RO depletion, and comparison of magnitude of responses to peptide stimulation.

Previous research has shown that T_{reg} cells are able to suppress $CD4^+$ T cell responses to CRC antigens 5T4 and CEA (Betts *et al*. 2012; Scurr *et al*. 2013; Besneux *et al*. 2019). This provided clear rationale for the depletion of T_{reg} cells in CRC patients using low-dose CPM in the TaCTiCC clinical trial. Results demonstrated that T_{reg} depletion was able to increase anti-tumour Th1 responses against tumour antigen 5T4. It would be worthwhile investigating the association with T_{reg} number on magnitude of response to the identified candidate antigens, in both healthy donors, and CRC patients. It is an attractive hypothesis that CPM treatment would increase responses to these antigens. While there is some evidence that this may indeed be true, due to experimental limitations it is vital this this be repeated in freshly isolated PBMC of additional patients receiving CPM treatment.

It has been shown that decreased Th1 responses to 5T4 are observed in CRC patients compared to healthy donors, and that these responses steadily decline during the advancement of disease (Scurr *et al*. 2013). To ascertain the association of reduced Th1 responses to CEACAM3, DNAJB7, and ZSWIM1 described in this thesis, increased numbers of CRC patients with a range of disease stages should be investigated to determine whether loss of response to these candidate antigens follows the same pattern as 5T4. Additionally, T cell responses could be further explored using epitope mapping techniques, and donors of known HLA-types. Given that several of the novel antigen candidates are described and increased in other malignancies, for example DNAJB7, it would be interesting to investigate antigen-specific T cell responses in patients with other types of cancer. This would assess the usefulness of immunotherapeutic targeting of such responses across multiple cancer types.

The results discussed in this Chapter have confirmed the immunogenicity of several novel tumour antigen candidates, identified through RNA sequencing of purified CRC tumours. Effector and memory Th1 responses have been demonstrated to several of

these candidates. Importantly, responses to CEACAM3, DNAJB7, and ZSWIM1 appear to be reduced in CRC patients compared to controls, therefore may represent viable CRC immunotherapy targets. The expression profile of DNAJB7 in particular shows limited background expression in healthy tissue, with the exception of testis. DNAJB7 may, therefore, represent a novel cancer-testis antigen. Further studies should be undertaken to confirm the immunogenicity of these proteins and to further assess the relationship of such responses to CRC staging. Background expression of target antigens in healthy tissue represents a major safety concern for cancer immunotherapy. Notably, affinity enhanced T cells against the MAGE-A3 antigen demonstrated cross-reactivity against titin, a protein present in muscle tissue. During testing, this led to cardiac arrest and subsequent death of 2 patients enrolled on the trial (Linette *et al*. 2013; Raman *et al*. 2016). The suitability of the candidates explored in this Chapter as targets for cancer immunotherapy should, therefore, be therefore, be investigated in detail, and targeting should be treated with caution.

5.4 Key Findings

Th1 responses to novel tumour antigen candidates CEACAM3, DNAJB7, and ZSWIM1 may be reduced in CRC patients compared to healthy donors. Differences are similar to those seen for known CRC tumour antigen, 5T4. Publicly available protein and RNA profiles have identified DNAJB7 as a novel cancer-testis antigen, with the ideal expression profile for immunotherapeutic targeting. The potential of these proteins for immunotherapy should be further explored.

1. Several novel tumour antigen candidates, identified by the Godkin/Gallimore group through RNA sequencing, have been confirmed as immunogenic in healthy donors and CRC patients.

2. *Ex vivo* IFNγ/GZMB responses to these candidate antigens are significantly altered between healthy donors and CRC patients, however, are generally higher in healthy donors.

3. T cell expansion through short-term culture with peptide enables the robust enumeration of low-frequency responses to the identified novel tumour antigen candidates. Future analyses should be performed using this methodology.

4. Cultured IFNγ responses to 5T4, DNAJB7, and ZSWIM1 are reduced in CRC patients compared to healthy donors. Loss of response to 5T4 and ZSWIM1

appear to associate with increased T stage in CRC, and there is some evidence this may also be the case for DNAJB7. General loss of cultured IFNγ response to CEACAM3 was seen in CRC patients compared to controls.

5. There is some evidence that CPM treatment may increase cultured IFNγ responses to CEACAM3, DNAJB7, and ZSWIM1, however there is no clear trend between patients.

6. DNAJB7 is highly expressed in several tumours, including CRC. Its expression in healthy tissue, however, is limited to the testis. DNAJB7 may represent a novel cancer-testis antigen target for cancer immunotherapy.

Chapter 6 Final Discussion

This thesis addressed the following research questions:

1. Can a panel of plasma proteins identify earlier-stage and metastatic CRC patients from both each other, and from healthy donors?

2. Can we identify biomarkers of immunotherapeutic response to both CPM and TroVax?

3. Can we identify novel antigen targets for CRC immunotherapy?

This discussion will summarise the key findings and will review how these may contribute to the understanding and future treatment of CRC.

6.1 Identification of Colorectal Cancer Patients and Metastasis

Current diagnosis of CRC involves patients undergoing a colonoscopy, during which regions of interest can be visually inspected and small biopsies taken for further investigation by histology. This procedure is time consuming and requires a specialist. Moreover, it is highly invasive, and is often poorly tolerated by the patient. Patients with CRC are also investigated for the presence of distant metastasis, typically by CT or MRI. This can be inconclusive, particularly when lesions are small. There is, therefore, a need for less invasive testing to identify patients with CRC, and those with distant metastasis. This could improve disease identification and help patients to be streamlined for further clinical investigation. Importantly, 5-year survival for patients with metastatic disease is only around 12%; earlier identification could improve this.

It was hypothesised that a panel of plasma proteins would be able to differentiate between individuals with mCRC, those with earlier-stage CRC, and healthy donors. This was investigated by measuring 31 proteins with previous associations with cancer and/or immunity in the plasma of donors from each group. The results of this investigation are detailed within Chapter 3 of this thesis. Overall, global serological changes were found in CRC patients compared to healthy donors, which were further emphasised in metastatic disease. Moreover, proteins were identified that were significantly altered between earlier-stage CRC patients, and those with metastatic disease.

In patients with mCRC, there were generally higher levels of plasma proteins involved in ECM remodelling, such as TIMP-1, Tenascin-C, and MMPs, and lower levels of immune-related proteins such as APOA1 and IL-2, compared to healthy donors. These changes were frequently pronounced between patients with earlier-stage disease and healthy controls/mCRC patients. The identified proteins may, therefore, represent pathways that become deregulated in CRC development and progression. It would be reasonable to hypothesise that increased plasma proteins associated with driving tumour progression, and reduced plasma proteins associated with immune activation, could be usefully employed to identify patients with earlier-stage and mCRC; this thesis supports that hypothesis.

The proteins found to be significantly altered between the three groups were assessed individually for their diagnostic potential using receiver operating characteristic (ROC) curve analysis. Individually, the best plasma protein for the identification of mCRC compared to healthy donors was Tenascin-C, with sensitivity and specificity of 75% and 90% respectively. Tenascin-C was also able to differentiate between earlier-stage and mCRC patients with sensitivity and specificity of 65% and 93% respectively. For the identification of earlier-stage CRC patients compared to mCRC patients, RANTES yielded a sensitivity and specificity of 83% and 64% respectively. These are arguably impressive results; however, it should be noted that Youden's index was used to determine the best cut-off to maximise both sensitivity and specificity. Either one of these outputs could be increased by sacrificing the other. Additionally, the earlier-stage CRC group was small, therefore the identified proteins may actually have improved diagnostic capacity in a larger cohort of patients.

It was clear from assessment of each protein individually that while impressive sensitivities and specificities were possible, to achieve 100% accuracy was unrealistic. There was overlap between these levels measured in healthy and diseased donors, even if significant overall increases/decreases were found. These proteins have fundamental physiological roles; therefore, overlap was expected. To improve the sensitivity and specificity of the plasma proteins for identifying each group, a combinatory approach was taken. Combination improved diagnostic accuracy compared to each protein alone, for example, the combination of APOA1, IL-2, Tenascin-C, and TIMP-1 by logistic regression was able to yield sensitivity and specificity of 84% and 90% respectively for the identification of mCRC patients compared to healthy donors.

Collectively, the findings described in Chapter 3 serve as proof of principle for the usefulness of plasma protein measurements for the identification of CRC. This is

particularly convincing for identifying mCRC patients compared to healthy controls, however, there is an indication that such measurements would also be useful for identifying earlier-stage CRC patients compared to both healthy donors and mCRC patients. The identified plasma proteins may, therefore, represent attractive candidates for CRC diagnostic testing. This should be investigated in a larger cohort of patients to further explore the possibility of a “blood test” for the identification of CRC.

Although ultimately visual confirmation will be required by colonoscopy, these measurements could help identify those individuals needing urgent referral and more detailed investigation. They may also have the potential to identify patients at risk of relapse who should be closely monitored; this possibility should be explored. Luminex multiplex is a novel methodology that allows for the simultaneous detection of several proteins in the same sample; it requires very little biological material which could be obtained as a by-product of common tests such as whole blood counts. It is simple, time and cost effective, and comparatively non-invasive compared to colonoscopy. Although the measurement of some of the described proteins, such as TIMP-1, have previously been proposed as individual diagnostic markers for CRC, the combinations described in this thesis are novel. Moreover, the described findings clearly support that improved diagnostic accuracy can be achieved by combining multiple relevant markers; this should be considered for the future development of diagnostic tests.

6.2 Prediction of Advanced Metastatic CRC Patient Response to Cyclophosphamide and TroVax

The Godkin/Gallimore group has recently demonstrated significantly increased survival in a proportion of advanced mCRC patients treated with CPM and/or TroVax during the TaCTiCC clinical trial (Scurr *et al*. 2017a; Scurr *et al*. 2017b). As a result, CPM and TroVax emerged as viable treatment options for mCRC, however, there is a lack of understanding as to which patients would most benefit from this intervention. It was hypothesised that a panel of pre-treatment plasma proteins, in addition to immunological and serological measurements taken by the NHS during routine blood testing, would be able to identify patients most likely to respond to each treatment. This was investigated retrospectively in the TaCTiCC cohort and is detailed within Chapter 4.

Several plasma proteins and immunological/serological measurements were significantly altered between patients who responded to CPM and those who did not including: $CD3^{+}CD4^{+}$ cell number, T_{regs}, WBCs, MMP-3, Tenascin-C, and TIMP-1. This suggests that pre-treatment immune function, in addition to the extent of ECM

remodelling/cancer progression, may be able to predict CPM treatment efficacy. Combinations of these measurements showed improved prediction of response than for each alone. Metronomic CPM is known to deplete T_{regs} (reviewed in Hughes *et al*. 2018). It could therefore be hypothesised that patients with increased numbers of T_{regs}/$CD3^+CD4^+$ cells may not "respond" to therapy as treatment simply cannot reduce cell numbers quickly enough to benefit these patients. It would be interesting to determine whether prolonged treatment or altered dosing would impact response to CPM. Moreover, it is possible that treating earlier-stage CRC with CPM may increase treatment efficacy. T_{reg} and $CD3^+CD4^+$ cell number were also able to identify patients with significantly reduced progression-free survival, supporting that tumour immunosuppression can drive tumour progression.

The finding that plasma MMP-3, Tenascin-C, and TIMP-1 are highest in patients who do not respond to CPM also suggests that these patients are perhaps too advanced to benefit from therapy. These proteins are involved in ECM remodelling and cancer metastasis. In addition to this, however, TIMP-1 is known to inhibit cellular apoptosis, and has been described as predictive of response to first-line cyclophosphamide/methotrexate/5-fluorouracil (CMF) and anthracycline-based chemotherapy in metastatic breast cancer patients (Schrohl *et al*. 2006; Klintman *et al*. 2010). It is possible, therefore, that TIMP-1 directly impedes upon response to CPM-based immunotherapy. This thesis has also identified a potential association between plasma TIMP-1 and the presence of liver metastasis. Given the requirement of CPM to be metabolised in the liver to become activated, impaired liver function as a result of metastasis may limit response. In CPM-treated patients, increased levels of both MMP-3 and TIMP-1 appear to relate to significantly worse progression-free survival. Moreover, TIMP-1 appears to also predict overall survival in the entire TaCTiCC cohort, with high levels associating with significantly reduced survival. Measurement of these proteins provides a wealth of information about CRC patient suitability for treatment and prognosis. Tenascin-C and TIMP-1 are significantly increased in CRC patients compared to healthy donors and are also significantly increased in mCRC patients compared to earlier-stage CRC patients; these proteins are markers of advanced disease. In addition to this, higher levels within the mCRC group identify patients who do not benefit from CPM treatment; perhaps these patients are beyond the stage of benefitting from immunotherapy. This observation strongly suggests that such treatments may be more effective in earlier stage disease. This will be investigated within the Godkin/Gallimore group through the "Brief Intervention with Cyclophosphamide in Patients with Colorectal Cancer" (BICCC) clinical trial, which will target earlier-stage CRC patients.

Pre-existing IFNγ T cell responses to 5T4 were significantly higher in patients who did not respond to TroVax treatment. In light of this finding, it would be reasonable to conclude than in order for immunotherapy to succeed, it must target aspects of immunity that have become deregulated in each patient. It is likely that non-responders did not respond as they were already mounting ineffective anti-tumour immune responses against 5T4. This finding strongly supports the transition towards personalised approaches to treat cancer; "one size fits all" approaches are no longer appropriate. Moreover, this finding supports that immunotherapies targeting several tumour antigens may be more efficacious than those targeting a single antigen. Plasma MMP-7 and CCL17 also emerged as potential biomarkers of TroVax response, with increased levels of MMP-7 and reduced levels of CCL17 found in TroVax non-responders. MMP-7 is a matrix protein involved in the remodelling of the ECM and has been linked with tumour progression, whereas CCL17 is a chemokine involved in T cell chemotaxis. There was an indication that patients with high CCL17 had higher overall survival, and those with high MMP-7 had significantly reduced overall and progression-free survival. These results collectively suggest that to achieve success with cancer vaccination, antigen selection is important. Moreover, immune functionality and extent of tumour progression/metastasis may impact efficacy, again supporting that the treatment of earlier-stage patients may improve outcomes.

6.3 Identification of Novel Antigen Targets for CRC Immunotherapy

Although the TaCTiCC trial demonstrated improved survival outcomes and increased responses to cancer antigen 5T4 in a proportion of mCRC patients, all patients had progressed within 10 months. This could be a result of increased immunosuppression associated with cancer progression; perhaps such therapies would be more efficacious in earlier-stage patients. Another reason for this could be a result of poor antigenic selection; perhaps 5T4 is not the optimal target. Moreover, it could be possible that targeting one antigen is simply not enough; not all tumour cells will express an antigen, even if a tumour is, for example, $5T4^{+}$.

To address this, the Godkin/Gallimore group identified 7 novel antigen candidates for CRC through purification and subsequent RNA sequencing of tumour and adjacent colon from 3 CRC patients. These candidates were significantly increased in tumour tissue of at least 2/3 patients. IFNγ T cell responses to 5T4 are reduced in patients with CRC compared to healthy donors, and this appears to be associated with tumour stage (Scurr *et al*. 2013). It was hypothesised that T cells responses to the

identified novel antigen candidates would follow this pattern. This thesis describes the investigation of *ex vivo* and cultured T cell responses in healthy donors and CRC patients to each of these potential antigens using pools of overlapping 20mer peptides.

Th1 T cell responses to CEACAM3, DNAJB7, and ZSWIM1 appeared to be reduced in CRC patients compared to healthy donors and showed a similar pattern as for 5T4; loss was exacerbated in patients with advancing tumour stage. These responses were low-frequency *ex vivo*, however, were readily expanded in culture, suggesting that they may represent $CD45RO^+$ memory T cell responses. These may, therefore, represent immunogenic antigens which become suppressed during CRC development. Loss of these responses in CRC patients is likely to reflect diminished anti-tumour immune responses and increased immunosuppression, which is well-described in tumorigenesis. In healthy donors, however, it is interesting that such vast memory T cell responses are demonstrated for self-antigens. This raises important questions as to why thymic selection has not deleted T cells specific for these antigens. It could be hypothesised that these T cells play a role in ongoing tumour immunosurveillance; this is supported by the observation that responses are diminished in cancer patients. Our group as shown that 5T4 is transiently upregulated on the colon of non-cancer patients with inflammatory bowel conditions (Scurr *et al*. 2013). It is possible, therefore, that T cell responses to 5T4 help to resolve periods of inflammation. This could also be true of novel tumour antigen candidates CEACAM3, DNAJB7, and ZSWIM1.

Our group has shown that T_{reg} depletion with CPM is able to increase IFNγ T cell responses to 5T4 in mCRC patients (Scurr *et al*. 2017a; Scurr *et al.* 2017b). It was therefore hypothesised that responses to CEACAM3, DNAJB7, and ZSWIM1 would also increase upon CPM treatment. This was investigated retrospectively in the same TaCTiCC cohort as for 5T4, using frozen PBMC. Although results were limited by cell number and viability, there was an indication that CPM may increase responses to these antigens in some patients. Preliminary experiments within the Godkin/Gallimore group using fresh PBMC have supported that CPM treatment can increase responses to DNAJB7; it is possible that regulatory T cells are reducing responses.

The expression profiles of the novel candidate tumour antigens were investigated using publicly available data on the Human Protein Atlas. An important consideration when targeting tumour associated antigens is the level of background expression on healthy tissues; this can lead to off-target side effects. The expression profile for DNAJB7 proposed it as a novel cancer-testis antigen, with lack of expression healthy tissues, with

the exception of testis and placenta. This represents the ideal expression profile for immunotherapeutic targeting.

The results of this investigation have identified potential CRC antigens, the responses to which are diminished during tumour development. This is likely to be a result of effective immune evasion by the tumour and may coincide with increased immunosuppression. Patients with reduced responses to a given antigen were more likely to also have reduced responses to other antigens; these patients may have increased T_{reg} numbers, or simply may not respond to these antigens. Targeting multiple tumour antigens may represent a more viable therapeutic strategy as it would, in theory, help to overcome inherent immune evasion and evolution. Moreover, antigens currently targeted by cancer vaccines, such as 5T4 and CEA, may lack the potency or expression profile to be effective. It is hoped that through the identification of additional potential CRC antigens, that more effective immunotherapies will be developed.

6.4 Concluding Remarks

Earlier and more efficient detection of CRC, combined with improved understanding of which patients are likely to benefit from targeted therapies, would improve patient survival. Identification of additional antigen targets for immunotherapy may be able to improve current therapies and overcome current limitations such as poor antigen selection. This thesis demonstrates clear advances to the improvement of CRC identification, patient treatment stratification, and immunotherapeutic targeting, albeit in small sample sizes. If these findings hold true in subsequent investigations, then improvements could be made to how we identify and treat patients with CRC.

While the findings described in this thesis provide proof of concept for the usefulness of plasma proteins in a diagnostic blood test for CRC, they also shed light onto the developmental process of CRC. The importance of both immunity and cancer-associated ECM remodelling for driving tumour progression has been validated and is sufficiently altered across CRC development to enable disease identification, and potentially disease staging. Moreover, these results demonstrate that the simple measurement of one plasma protein, for example TIMP-1, is able to provide a wealth of clinical information, including: an impressive prediction of presence of CRC and metastasis, the likelihood of response to CPM-based immunotherapy, the presence of liver metastasis, and a projection of overall and progression-free survival. This can be further improved upon by considering additional proteins.

An important conclusion to be drawn from this thesis is that in order for immunotherapy to succeed, antigen targets must be appropriate. Furthermore, targeting patients earlier is likely to improve responses to immunotherapy. The improved identification of CRC patients, better treatment targeting, and treatment success in early-stage clinical trials may help this to become a reality.

Through earlier/improved detection, better treatment stratification, and the identification of additional antigen targets, it is hoped that treatment and survival of CRC patients will be improved. This thesis summarises novel preliminary advances made towards each of these goals.

Appendix

(A)Antigen peptides and peptide pool configuration.

Supplementary Table 1.1 ARSJ Peptides

58 20mers, 1 19mer, overlapping by 10 amino acids. Split into 3 peptide pools.
Peptide Pool 1 = Peptides 1-19
Peptide Pool 2 = Peptides 20-38
Peptide Pool 3 = Peptides 39-59

1) MAPRGCAGHPPPPSPQACVC
2) PPPSPQACVCPGKMLAMGAL
3) PGKMLAMGALAGFWILCLLT
4) AGFWILCLLTYGYLSWGQAL
5) YGYLSWGQALEEEEEGALLA
6) EEEEEGALLAQAGEKLEPST
7) QAGEKLEPSTTSTSQPHLIF
8) TSTSQPHLIFILADDQGFRD
9) ILADDQGFRDVGYHGSEIKT
10) VGYHGSEIKTPTLDKLAAEG
11) PTLDKLAAEGVKLENYYVQP
12) VKLENYYVQPICTPSRSQFI
13) ICTPSRSQFITGKYQIHTGL
14) TGKYQIHTGLQHSIIRPTQP
15) QHSIIRPTQPNCLPLDNATL
16) NCLPLDNATLPQKLKEVGYS
17) PQKLKEVGYSTHMVGKWHLG
18) THMVGKWHLGFYRKECMPTR
19) FYRKECMPTRRGFDTFFGSL

20) RGFDTFFGSLLGSGDYYTHY
21) LGSGDYYTHYKCDSPGMCGY
22) KCDSPGMCGYDLYENDNAAW
23) DLYENDNAAWDYDNGIYSTQ
24) DYDNGIYSTQMYTQRVQQIL
25) MYTQRVQQILASHNPTKPIF
26) ASHNPTKPIFLYIAYQAVHS

27) LYIAYQAVHSPLQAPGRYFE
28) PLQAPGRYFEHYRSIININR
29) HYRSIININRRRYAAMLSCL
30) RRYAAMLSCLDEAINNVTLA
31) DEAINNVTLALKTYGFYNNS
32) LKTYGFYNNSIIIYSSDNGG
33) IIIYSSDNGGQPTAGGSNWP
34) QPTAGGSNWPLRGSKGTYWE
35) LRGSKGTYWEGGIRAVGFVH
36) GGIRAVGFVHSPLLKNKGTV
37) SPLLKNKGTVCKELVHITDW
38) CKELVHITDWYPTLISLAEG

39) YPTLISLAEGQIDEDIQLDG
40) QIDEDIQLDGYDIWETISEG
41) YDIWETISEGLRSPRVDILH
42) LRSPRVDILHNIDPIYTKAK
43) NIDPIYTKAKNGSWAAGYGI
44) NGSWAAGYGIWNTAIQSAIR
45) WNTAIQSAIRVQHWKLLTGN
46) VQHWKLLTGNPGYSDWVPPQ
47) PGYSDWVPPQSFSNLGPNRW
48) SFSNLGPNRWHNERITLSTG
49) HNERITLSTGKSVWLFNITA
50) KSVWLFNITADPYERVDLSN
51) DPYERVDLSNRYPGIVKKLL
52) RYPGIVKKLLRRLSQFNKTA
53) RRLSQFNKTAVPVRYPPKDP
54) VPVRYPPKDPRSNPRLNGGV
55) RSNPRLNGGVWGPWYKEETK
56) WGPWYKEETKKKKPSKNQAE
57) KKKPSKNQAEKKQKKSKKKK
58) KKQKKSKKKKKKQQKAVSGS
59) KKQQKAVSGSTCHSGVTCG

Supplementary Table 1.2 CEACAM3 Peptides

23 20mers, 1 22mer, overlapping by 10 amino acids. Split into 2 peptide pools.
Peptide Pool 1 = Peptides 1-12
Peptide Pool 2 = Peptides 13-24

1) MGPPSASPHRECIPWQGLLL
2) ECIPWQGLLLTASLLNFWNP
3) TASLLNFWNPPTTAKLTIES
4) PTTAKLTIESMPLSVAEGKE
5) MPLSVAEGKEVLLLVHNLPQ
6) VLLLVHNLPQHLFGYSWYKG
7) HLFGYSWYKGERVDGNSLIV
8) ERVDGNSLIVGYVIGTQQAT
9) GYVIGTQQATPGAAYSGRET
10) PGAAYSGRETIYTNASLLIQ
11) IYTNASLLIQNVTQNDIGFY
12) NVTQNDIGFYTLQVIKSDLV

13) TLQVIKSDLVNEEATGQFHV
14) NEEATGQFHVYQENAPGLPV
15) YQENAPGLPVGAVAGIVTGV
16) GAVAGIVTGVLVGVALVAAL
17) LVGVALVAALVCFLLLAKTG
18) VCFLLLAKTGRTSIQRDLKE
19) RTSIQRDLKEQQPQALAPGR
20) QQPQALAPGRGPSHSSAFSM
21) GPSHSSAFSMSPLSTAQAPL
22) SPLSTAQAPLPNPRTAASIY
23) PNPRTAASIYEELLKHDTNI
24) EELLKHDTNIYCRMDHKAEVAS

Supplementary Table 1.3 CENPQ Peptides

25 20mers, 1 18mer, overlapping by 10 amino acids. Split into 2 peptide pools.
Peptide Pool 1 = Peptides 1-13
Peptide Pool 2 = Peptides 14-26

1) MSGKANASKKNAQQLKRNPK
2) NAQQLKRNPKRKKDNEEVVL
3) RKKDNEEVVLSENKVRNTVK
4) SENKVRNTVKKNKNHLKDLS
5) KNKNHLKDLSSEGQTKHTNL
6) SEGQTKHTNLKHGKTAASKR
7) KHGKTAASKRKTWQPLSKST
8) KTWQPLSKSTRDHLQTMMES
9) RDHLQTMMESVIMTILSNSI
10) VIMTILSNSIKEKEEIQYHL
11) KEKEEIQYHLNFLKKRLLQQ
12) NFLKKRLLQQCETLKVPPKK
13) CETLKVPPKKMEDLTNVSSL

14) MEDLTNVSSLLNMERARDKA
15) LNMERARDKANEEGLALLQE
16) NEEGLALLQEEIDKMVETTE
17) EIDKMVETTELMTGNIQSLK
18) LMTGNIQSLKNKIQILASEV
19) NKIQILASEVEEEEERVKQM
20) EEEEERVKQMHQINSSGVLS
21) HQINSSGVLSLPELSQKTLK
22) LPELSQKTLKAPTLQKEILA
23) APTLQKEILALIPNQNALLK
24) LIPNQNALLKDLDILHNSSQ
25) DLDILHNSSQMKSMSTFIEE
26) MKSMSTFIEEAYKKLDAS

Supplementary Table 1.4 CYP2B6 Peptides

47 20mers, 1 21mer, overlapping by 10 amino acids. Split into 3 peptide pools.
Peptide Pool 1 = Peptides 1-16
Peptide Pool 2 = Peptides 17-32
Peptide Pool 3 = Peptides 33-48

1) MELSVLLFLALLTGLLLLLV
2) LLTGLLLLLVQRHPNTHDRL
3) QRHPNTHDRLPPGPRPLPLL
4) PPGPRPLPLLGNLLQMDRRG
5) GNLLQMDRRGLLKSFLRFRE
6) LLKSFLRFREKYGDVFTVHL
7) KYGDVFTVHLGPRPVVMLCG
8) GPRPVVMLCGVEAIREALVD
9) VEAIREALVDKAEAFSGRGK
10) KAEAFSGRGKIAMVDPFFRG
11) IAMVDPFFRGYGVIFANGNR
12) YGVIFANGNRWKVLRRFSVT
13) WKVLRRFSVTTMRDFGMGKR
14) TMRDFGMGKRSVEERIQEEA
15) SVEERIQEEAQCLIEELRKS
16) QCLIEELRKSKGALMDPTFL

17) KGALMDPTFLFQSITANIIC
18) FQSITANIICSIVFGKRFHY
19) SIVFGKRFHYQDQEFLKMLN
20) QDQEFLKMLNLFYQTFSLIS
21) LFYQTFSLISSVFGQLFELF
22) SVFGQLFELFSGFLKYFPGA
23) SGFLKYFPGAHRQVYKNLQE
24) HRQVYKNLQEINAYIGHSVE
25) INAYIGHSVEKHRETLDPSA
26) KHRETLDPSAPKDLIDTYLL
27) PKDLIDTYLLHMEKEKSNAH
28) HMEKEKSNAHSEFSHQNLNL
29) SEFSHQNLNLNTLSLFFAGT
30) NTLSLFFAGTETTSTTLRYG

31) ETTSTTLRYGFLLMLKYPHV
32) FLLMLKYPHVAERVYREIEQ

33) AERVYREIEQVIGPHRPPEL
34) VIGPHRPPELHDRAKMPYTE
35) HDRAKMPYTEAVIYEIQRFS
36) AVIYEIQRFSDLLPMGVPHI
37) DLLPMGVPHIVTQHTSFRGY
38) VTQHTSFRGYIIPKDTEVFL
39) IIPKDTEVFLILSTALHDPH
40) ILSTALHDPHYFEKPDAFNP
41) YFEKPDAFNPDHFLDANGAL
42) DHFLDANGALKKTEAFIPFS
43) KKTEAFIPFSLGKRICLGEG
44) LGKRICLGEGIARAELFLFF
45) IARAELFLFFTTILQNFSMA
46) TTILQNFSMASPVAPEDIDL
47) SPVAPEDIDLTPQECGVGKI
48) TPQECGVGKIPPTYQIRFLPR

Supplementary Table 1.5 DNAJB7 Peptides

29 20mers, 1 19mer, overlapping by 10 amino acids. Split into 2 peptide pools.
Peptide Pool 1 = Peptides 1-15
Peptide Pool 2 = Peptides 16-30

1) MVDYYEVLGLQRYASPEDIK
2) QRYASPEDIKKAYHKVALKW
3) KAYHKVALKWHPDKNPENKE
4) HPDKNPENKEEAERKFKEVA
5) EAERKFKEVAEAYEVLSNDE
6) EAYEVLSNDEKRDIYDKYGT
7) KRDIYDKYGTEGLNGGGSHF
8) EGLNGGGSHFDDECEYGFTF
9) DDECEYGFTFHKPDDVFKEI
10) HKPDDVFKEIFHERDPFSFH
11) FHERDPFSFHFFEDSLEDLL
12) FFEDSLEDLLNRPGSSYGNR
13) NRPGSSYGNRNRDAGYFFST
14) NRDAGYFFSTASEYPIFEKF
15) ASEYPIFEKFSSYDTGYTSQ

16) SSYDTGYTSQGSLGHEGLTS
17) GSLGHEGLTSFSSLAFDNSG
18) FSSLAFDNSGMDNYISVTTS
19) MDNYISVTTSDKIVNGRNIN
20) DKIVNGRNINTKKIIESDQE
21) TKKIIESDQEREAEDNGELT
22) REAEDNGELTFFLVNSVANE
23) FFLVNSVANEEGFAKECSWR
24) EGFAKECSWRTQSFNNYSPN
25) TQSFNNYSPNSHSSKHVSQY
26) SHSSKHVSQYTFVDNDEGGI
27) TFVDNDEGGISWVTSNRDPP
28) SWVTSNRDPPIFSAGVKEGG
29) IFSAGVKEGGKRKKKKRKEV
30) KRKKKKRKEVQKKSTKRNC

Supplementary Table 1.6 ZC3H12B Peptides

82 20mers, 1 16mer, overlapping by 10 amino acids. Split into 3 peptide pools.
Peptide Pool 1 = Peptides 1-27
Peptide Pool 2 = Peptides 28-55
Peptide Pool 3 = Peptides 56-83

1) MTATAEVETPKMEKSASKEE
2) KMEKSASKEEKQQPKQDSTE
3) KQQPKQDSTEQGNADSEEWM
4) QGNADSEEWMSSESDPEQIS
5) SSESDPEQISLKSSDNSKSC
6) LKSSDNSKSCQPRDGQLKKK
7) QPRDGQLKKKEMHSKPHRQL
8) EMHSKPHRQLCRSPCLDRPS
9) CRSPCLDRPSFSQSSILQDG
10) FSQSSILQDGKLDLEKEYQA
11) KLDLEKEYQAKMEFALKLGY
12) KMEFALKLGYAEEQIQSVLN
13) AEEQIQSVLNKLGPESLIND
14) KLGPESLINDVLAELVRLGN
15) VLAELVRLGNKGDSEGQINL
16) KGDSEGQINLSLLVPRGPSS
17) SLLVPRGPSSREIASPELSL
18) REIASPELSLEDEIDNSDNL
19) EDEIDNSDNLRPVVIDGSNV
20) RPVVIDGSNVAMSHGNKEEF
21) AMSHGNKEEFSCRGIQLAVD
22) SCRGIQLAVDWFLDKGHKDI
23) WFLDKGHKDITVFVPAWRKE
24) TVFVPAWRKEQSRPDAPITD
25) QSRPDAPITDQDILRKLEKE
26) QDILRKLEKEKILVFTPSRR
27) KILVFTPSRRVQGRRVVCYD

28) VQGRRVVCYDDRFIVKLAFD
29) DRFIVKLAFDSDGIIVSNDN
30) SDGIIVSNDNYRDLQVEKPE

31) YRDLQVEKPEWKKFIEERLL
32) WKKFIEERLLMYSFVNDKFM
33) MYSFVNDKFMPPDDPLGRHG
34) PPDDPLGRHGPSLENFLRKR
35) PSLENFLRKRPIVPEHKKQP
36) PIVPEHKKQPCPYGKKCTYG
37) CPYGKKCTYGHKCKYYHPER
38) HKCKYYHPERANQPQRSVAD
39) ANQPQRSVADELRISAKLST
40) ELRISAKLSTVKTMSEGTLA
41) VKTMSEGTLAKCGTGMSSAK
42) KCGTGMSSAKGEITSEVKRV
43) GEITSEVKRVAPKRQSDPSI
44) APKRQSDPSIRSVAMEPEEW
45) RSVAMEPEEWLSIARKPEAS
46) LSIARKPEASSVPSLVTALS
47) SVPSLVTALSVPTIPPPKSH
48) VPTIPPPKSHAVGALNTRSA
49) AVGALNTRSASSPVPGSSHF
50) SSPVPGSSHFPHQKASLEHM
51) PHQKASLEHMASMQYPPILV
52) ASMQYPPILVTNSHGTPISY
53) TNSHGTPISYAEQYPKFESM
54) AEQYPKFESMGDHGYYSMLG
55) GDHGYYSMLGDFSKLNINSM

56) DFSKLNINSMHNREYYMAEV
57) HNREYYMAEVDRGVYARNPN
58) DRGVYARNPNLCSDSRVSHT
59) LCSDSRVSHTRNDNYSSYNN
60) RNDNYSSYNNVYLAVADTHP
61) VYLAVADTHPEGNLKLHRSA
62) EGNLKLHRSASQNRLQPFPH
63) SQNRLQPFPHGYHEALTRVQ
64) GYHEALTRVQSYGPEDSKQG
65) SYGPEDSKQGPHKQSVPHLA
66) PHKQSVPHLALHAQHPSTGT
67) LHAQHPSTGTRSSCPADYPM

68) RSSCPADYPMPPNIHPGATP
69) PPNIHPGATPQPGRALVMTR
70) QPGRALVMTRMDSISDSRLY
71) MDSISDSRLYESNPVRQRRP
72) ESNPVRQRRPPLCREQHASW
73) PLCREQHASWDPLPCTTDSY
74) DPLPCTTDSYGYHSYPLSNS
75) GYHSYPLSNSLMQPCYEPVM
76) LMQPCYEPVMVRSVPEKMEQ
77) VRSVPEKMEQLWRNPWVGMC
78) LWRNPWVGMCNDSREHMIPE
79) NDSREHMIPEHQYQTYKNLC
80) HQYQTYKNLCNIFPSNIVLA
81) NIFPSNIVLAVMEKNPHTAD
82) VMEKNPHTADAQQLAALIVA
83) AQQLAALIVAKLRAAR

Supplementary Table 1.7 ZSWIM1 Peptides

47 20mers, 1 15mer, overlapping by 10 amino acids. Split into 3 peptide pools.
Peptide Pool 1 = Peptides 1-16
Peptide Pool 2 = Peptides 17-32
Peptide Pool 3 = Peptides 33-48

1) MLERLKAPWSAALQRKYFDL
2) AALQRKYFDLGIWTAPISPM
3) GIWTAPISPMALTMLNGLLI
4) ALTMLNGLLIKDSSPPMLLH
5) KDSSPPMLLHQVNKTAQLDT
6) QVNKTAQLDTFNYQSCFMQS
7) FNYQSCFMQSVFDHFPEILF
8) VFDHFPEILFIHRTYNPRGK
9) IHRTYNPRGKVLYTFLVDGP
10) VLYTFLVDGPRVQLEGHLAR
11) RVQLEGHLARAVYFAIPAKE
12) AVYFAIPAKEDTEGLAQMFQ
13) DTEGLAQMFQVFKKFNPAWE
14) VFKKFNPAWERVCTILVDPH
15) RVCTILVDPHFLPLPILAME
16) FLPLPILAMEFPTAEVLLSA

17) FPTAEVLLSAFHICKFLQAK
18) FHICKFLQAKFYQLSLERPV
19) FYQLSLERPVERLLLTSLQS
20) ERLLLTSLQSTMCSATAGNL
21) TMCSATAGNLRKLYTLLSNC
22) RKLYTLLSNCIPPAKLPELH
23) IPPAKLPELHSHWLLNDRIW
24) SHWLLNDRIWLAHRWRSRAE
25) LAHRWRSRAESSHYFQSLEV
26) SSHYFQSLEVTTHILSQFFG
27) TTHILSQFFGTTPSEKQGMA
28) TTPSEKQGMASLFRYMQQNS
29) SLFRYMQQNSADKANFNQGL
30) ADKANFNQGLCAQNNHAPSD

31) CAQNNHAPSDTIPESPKLEQ
32) TIPESPKLEQLVESHIQHSL

33) LVESHIQHSLNAICTGPAAQ
34) NAICTGPAAQLCLGELAVVQ
35) LCLGELAVVQKSTHLIGSGS
36) KSTHLIGSGSEKMNIQILED
37) EKMNIQILEDTHKVQPQPPA
38) THKVQPQPPASCSCYFNQAF
39) SCSCYFNQAFHLPCRHILAM
40) HLPCRHILAMLSARRQVLQP
41) LSARRQVLQPDMLPAQWTAG
42) DMLPAQWTAGCATSLDSILG
43) CATSLDSILGSKWSETLDKH
44) SKWSETLDKHLAVTHLTEEV
45) LAVTHLTEEVGQLLQHCTKE
46) GQLLQHCTKEEFERRYSTLR
47) EFERRYSTLRELADSWIGPY
48) ELADSWIGPYEQVQL

Supplementary Table 1.8 5T4 Peptides

41 20mers, overlapping by 10 amino acids. Split into 2 peptide pools.
Peptide Pool 1 = Peptides 1-20
Peptide Pool 2 = Peptides 21-41

1) MPGGCSRGPAAGDGRLRLAR
2) AGDGRLRLARLALVLLGWVS
3) LALVLLGWVSSSSPTSSASS
4) SSSPTSSASSFSSSAPFLAS
5) FSSSAPFLASAVSAQPPLPD
6) AVSAQPPLPDQCPALCECSE
7) QCPALCECSEAARTVKCVNR
8) AARTVKCVNRNLTEVPTDLP
9) NLTEVPTDLPAYVRNLFLTG
10) AYVRNLFLTGNQLAVLPAGA
11) NQLAVLPAGAFARRPPLAEL
12) FARRPPLAELAALNLSGSRL
13) AALNLSGSRLDEVRAGAFEH
14) DEVRAGAFEHLPSLRQLDLS
15) LPSLRQLDLSHNPLADLSPF
16) HNPLADLSPFAFSGSNASVS
17) AFSGSNASVSAPSPLVELIL
18) APSPLVELILNHIVPPEDER
19) NHIVPPEDERQNRSFEGMVV
20) QNRSFEGMVVAALLAGRALQ

21) AALLAGRALQGLRRLELASN
22) GLRRLELASNHFLYLPRDVL
23) HFLYLPRDVLAQLPSLRHLD
24) AQLPSLRHLDLSNNSLVSLT
25) LSNNSLVSLTYVSFRNLTHL
26) YVSFRNLTHLESLHLEDNAL
27) ESLHLEDNALKVLHNGTLAE
28) KVLHNGTLAELQGLPHIRVF
29) LQGLPHIRVFLDNNPWVCDC
30) LDNNPWVCDCHMADMVTWLK
31) HMADMVTWLKETEVVQGKDR

32) ETEVVQGKDRLTCAYPEKMR

33) LTCAYPEKMRNRVLLELNSA

34) NRVLLELNSADLDCDPILPP

35) DLDCDPILPPSLQTSYVFLG

36) SLQTSYVFLGIVLALIGAIF

37) IVLALIGAIFLLVLYLNRKG

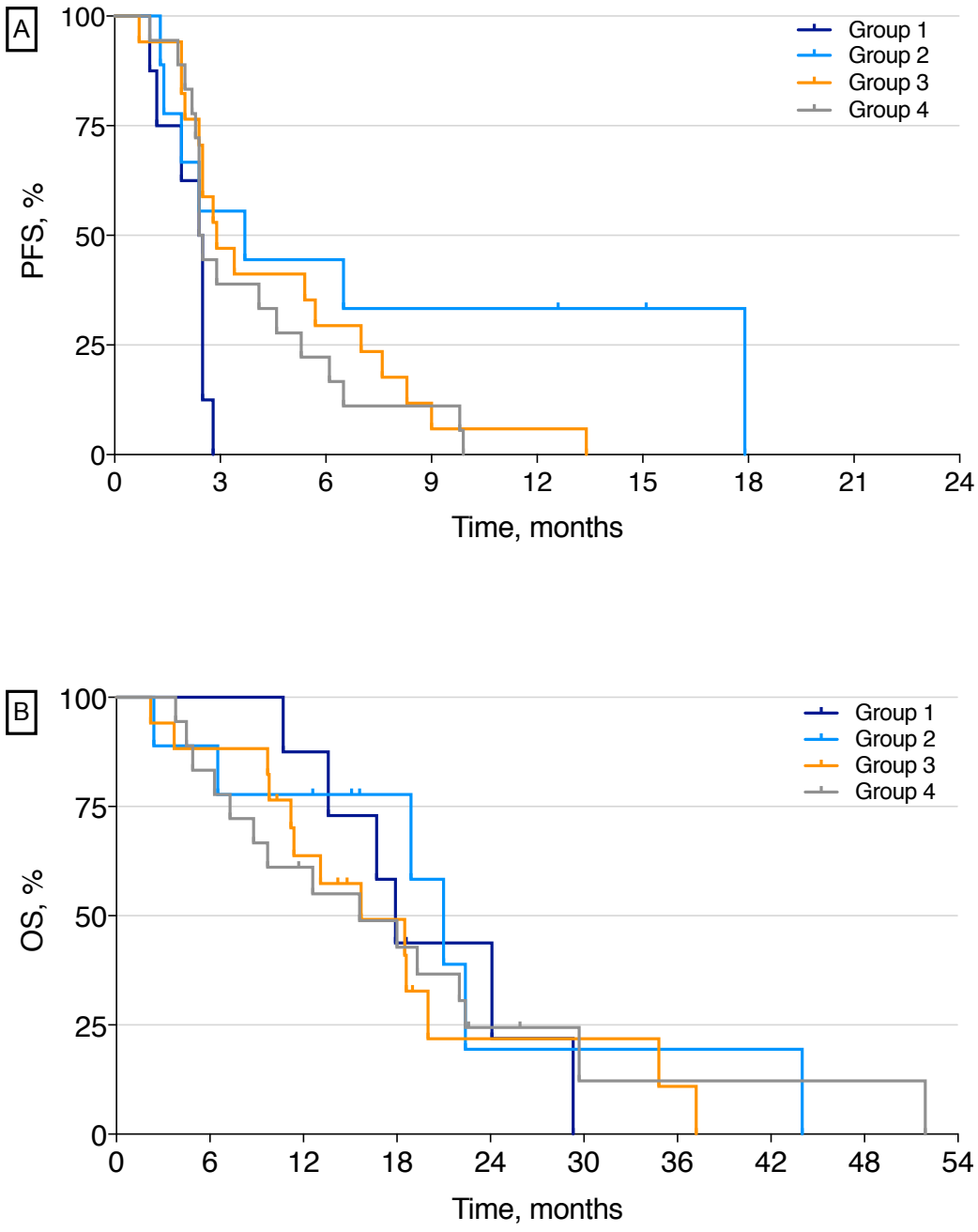
38) LLVLYLNRKGIKKWMHNIRD

39) IKKWMHNIRDACRDHMEGYH

40) ACRDHMEGYHYRYEINADPR

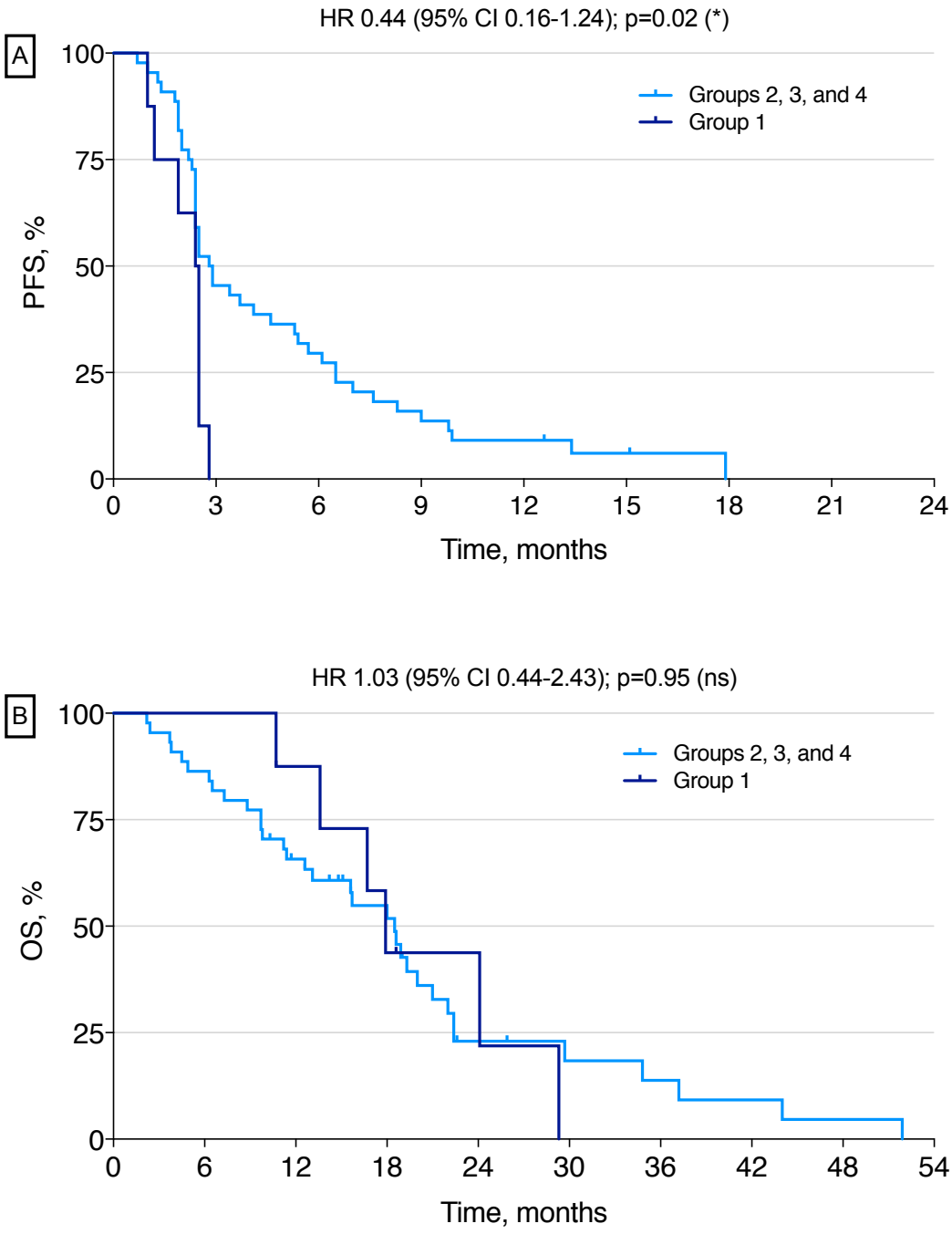
41) YRYEINADPRLTNLSSNSDV

(B) Survival outcomes from TaCTiCC



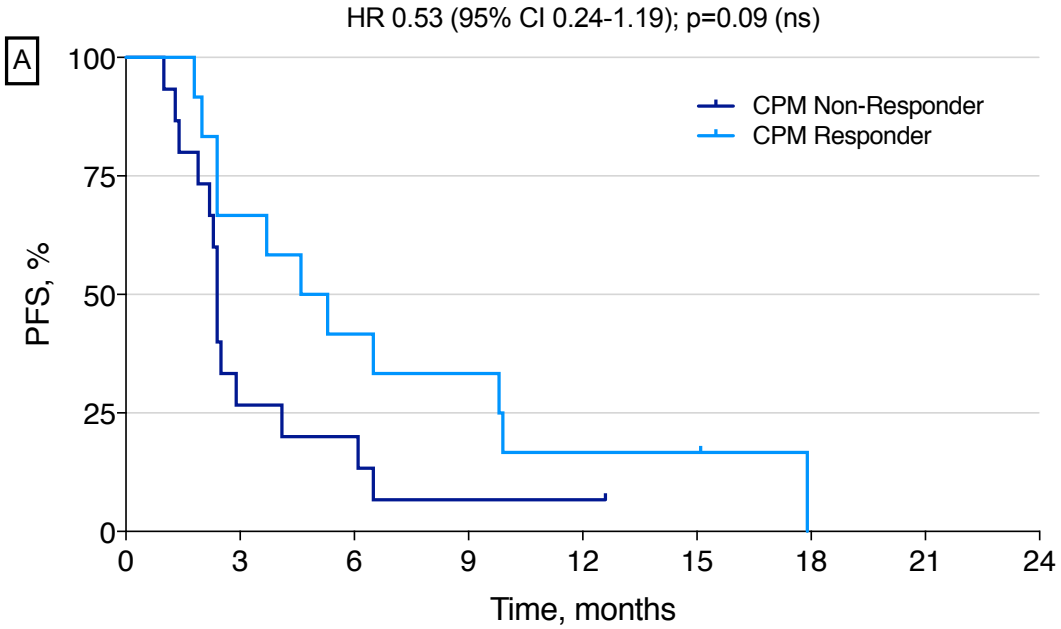
Supplementary Figure 1 (A-B) Therapeutic intervention with CPM/TroVax results in increased PFS survival in patients with mCRC.

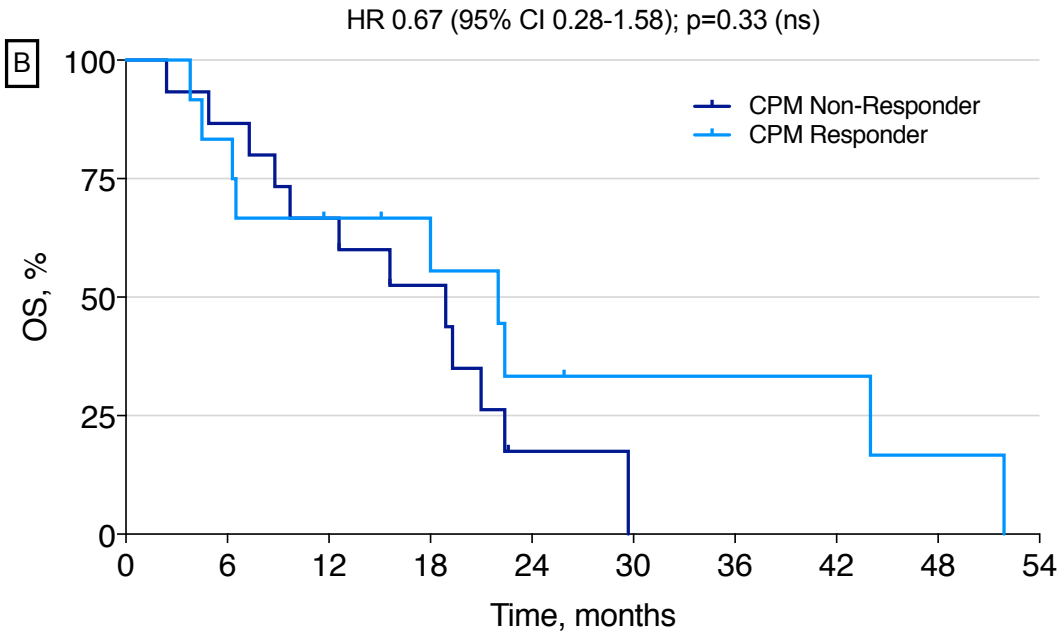
The progression-free (A) and overall survival (B) of patients within each group of TaCTiCC. Group 1 received no treatment (n=8), Group 2 received CPM (n=9), Group 3 received TroVax (n=17), and Group 4 received CPM and TroVax (n=18). Results are displayed using Kaplan-Meier plots. Adapted from (Scurr et al. 2017a).



Supplementary Figure 2 (A-B) Therapeutic intervention results in increased PFS survival in patients with mCRC.

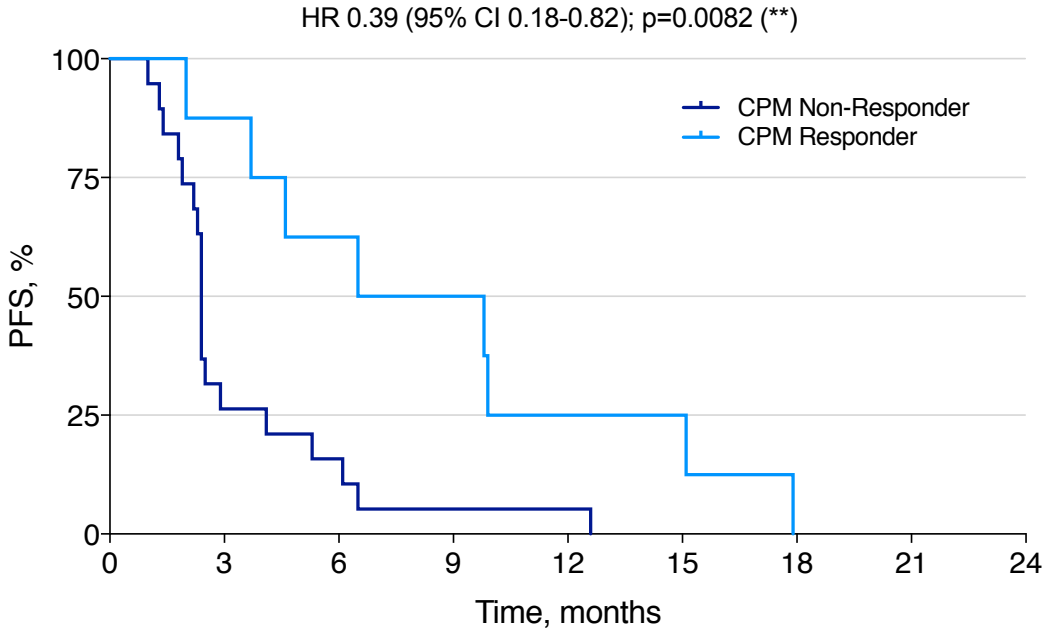
The progression-free (A) and overall survival (B) of patients receiving TroVax and/or CPM (Groups 2-4, n=44) compared to that of non-treatment controls (Group 1, n=8) by log-rank Mantel-Cox test. Results are displayed using Kaplan-Meier plots. Hazard ratio, confidence intervals, and p values are as shown. Adapted from (Scurr et al. 2017a).





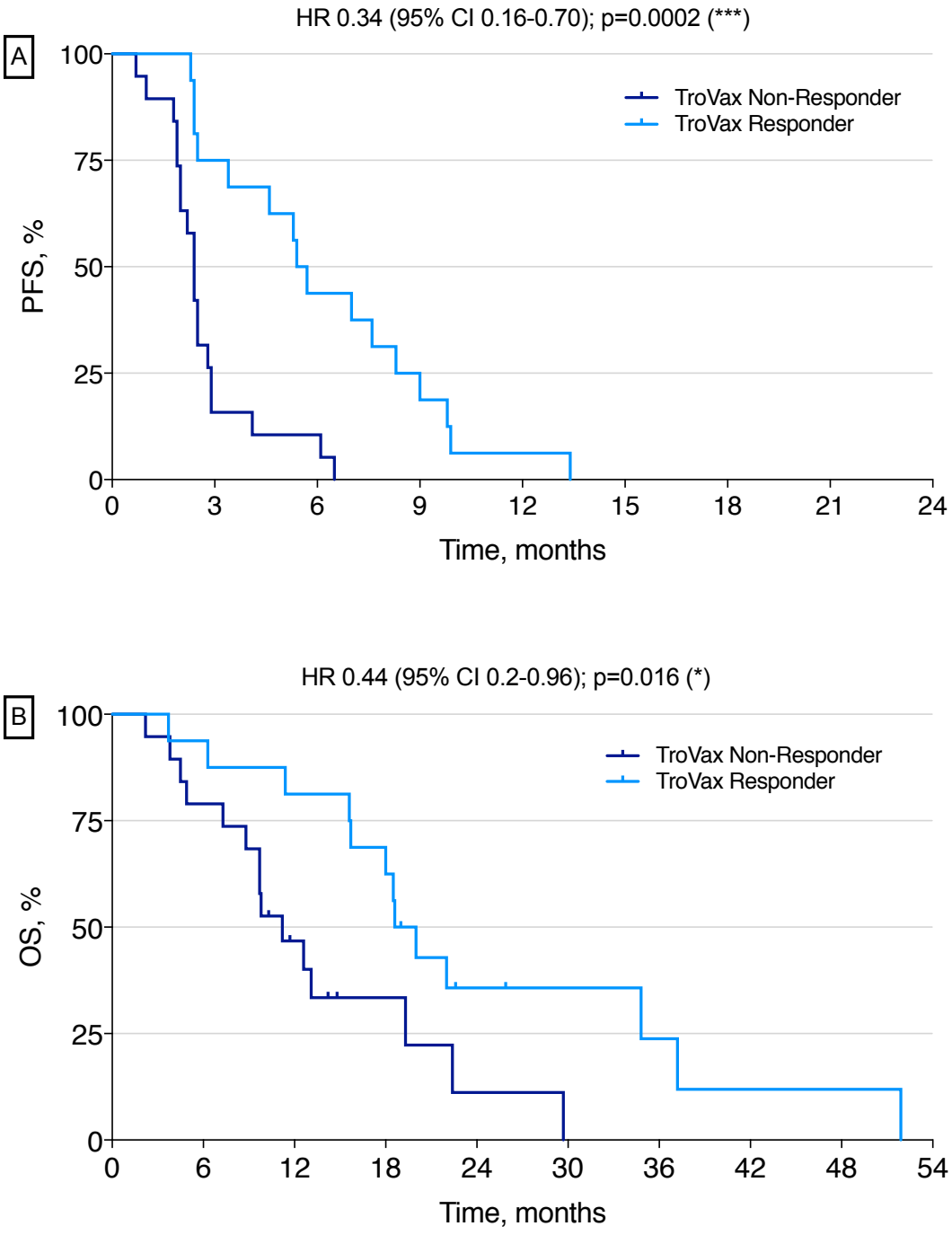
Supplementary Figure 3 (A-B) Immunological responders to CPM treatment show a trend for increased progression-free survival.

The progression-free (A) and overall survival (B) of patients receiving CPM treatment were split into those who responded to treatment (n=12) and those who did not (n=15), based on magnitude of T_{reg} depletion. Adapted from (Scurr et al. 2017a).



Supplementary Figure 4 CPM-treated patients who both decrease T_{reg} number, and increase IFNγ response to 5T4, have significantly increased progression-free survival.

The progression-free survival of patients receiving CPM treatment were split into those who responded to treatment (n=12) and those who did not (n=15), based on responders being those who both decreased their T_{reg} number and increased their IFNγ response to 5T4 (n=8), compared to those who either only responded by one measurement, or by neither (n=19). Adapted from (Scurr et al. 2017a).



Supplementary Figure 5 (A-B) Immunological responders to TroVax treatment show increased progression-free and overall survival.

The progression-free (A) and overall survival (B) of patients receiving TroVax treatment were split into those who responded to treatment (n=16) and those who did not (n=19) based on magnitude of IFNγ response to 5T4, and 5T4 antibody response. Adapted from (Scurr et al. 2017a).

(C*) Overview of the association of plasma proteins measured by Luminex with cancer/tumour immunity.*

Analyte	Biological Function	Association with Cancer/Tumour-Immunology	References
APOA1	Major serum protein component of high-density lipoprotein	Decreased serum levels associated with TNM stage, and decreased survival in CRC. Predictive of response in RCC patients treated with multi-peptide cancer vaccine and CPM	(Walter *et al*. 2012; Zamanian-Daryoush *et al*. 2013; Zamanian-Daryoush and DiDonato 2015; Sirniö *et al*. 2017)
BDNF	Nerve growth factor. Promotes brain neuronal survival. Decreased levels are associated with neurodegenerative diseases	Serum levels are decreased in CRC patients compared to healthy controls. Local levels within the tumour are increased compared to healthy tissue. Hypothalamic overexpression can decrease the ratio of CD4:CD8 T cells in mouse melanoma models	(Akil *et al*. 2011; Roesler *et al*. 2011; Brierley *et al*. 2013; Yang *et al*. 2013; Xiao *et al*. 2016; Radin and Parth 2017)

BTLA (HVEM)	Member of the immunoglobulin protein family. Acts as a receptor, mediating immune suppression	Inhibits function of cancer specific $CD8^+$ T cells. Upregulated alongside PD-1 on $CD4^+$ T cell from HCC patients. Expression within CRC tumours associated with tumour/pathological stage, reduced TILs, and reduced survival	(Derré *et al*. 2010; Inoue *et al*. 2015; Zhao *et al*. 2016)
CCL17 (TARC)	Secreted cytokine. Binds to CCR4 and CCR8 on T cells, inducing chemotaxis. Also aids T cell development in the thymus	Increased levels in RCC patients with increased survival, and who response to multi-peptide cancer vaccine, and CPM. Higher levels were associated with increased survival in advanced melanoma patients	(Walter *et al*. 2012; Weide *et al*. 2015)
CD27	Member of the TNF-receptor family. Binds to its ligand; CD70. T cell costimulatory molecule involved in the generation, and maintenance of long-term T cell immunity	Increased serum levels are associated with treatment outcome, increased survival in patients with large-cell B lymphoma, and in prostate cancer patients treated with immunotherapy	(Hendriks *et al*. 2000; Goto *et al*. 2012; Huang *et al*. 2013)
Eotaxin-1 (CCL11)	Cytokine involved in eosinophil chemotaxis. Binds to receptors CCR2, CCR3, and CCR5, found on the surface of multiple immune cell types	Serum levels of CCL11 are significantly elevated in prostate cancer patients, and is described as a marker of epithelial ovarian, and gastric cancers	(Zohny and Fayed 2010; Agarwal *et al.* 2013; Koç *et al*. 2013; Zhu *et al*. 2014)

HGF	Growth factor. Binds to hepatocyte growth factor receptor, MET. Regulates cell growth, motility, and differentiation, and also plays a role in angiogenesis and tissue repair. Secreted by mesenchymal cells	High serum levels are significantly associated with reduced survival in CRC. Can confer immune suppression, by inhibiting dendritic cell function	(Ziche *et al*. 1992; Okunishi *et al*. 2005; Toiyama *et al*. 2009; Matsumoto *et al*. 2017; Papaccio *et al*. 2018)
IDO	Intracellular enzyme involved in the kynurenine pathway, which produces nicotinamide adenine dinucleotide from the degradation of tryptophan. Reduction in tryptophan levels impairs T cell growth. IDO is induced by type-I and type-II interferons	Confers immune suppression by depleting T cells. Is considered a cancer immune checkpoint	(Huang *et al*. 2002; Munn and Mellor 2004; Mellor *et al*. 2017)
IL-15	Cytokine. Regulates NK and T cell activation and proliferation	Can increase the antitumor activity of $CD8^+$ T cells. Reduced IL-15 expression corresponds with reduced survival in CRC	(Klebanoff *et al*. 2004; Steel *et al*. 2012; Mlecnik *et al*. 2014)
IL-2	Cytokine. Promotes the development and proliferation of T_{reg} cells. Also promotes the development of effector and memory T cells during antigen presentation	Use as a monotherapy has been largely ineffective at prolonging patient survival in RCC and metastatic melanoma. Treatment leads to increased T_{regs}	(Nelson 2004; Jiang *et al*. 2016; Ahmadzadeh and Rosenberg 2018)

IL-21	Cytokine. Induces proliferation, and differentiation in multiple immune cell types, including B cells, NK cells, and various subtypes of T cell. Can also act as an immune-suppressor by inducing IL-10 production	Known to play a role in the regulation of colitis-associated colon cancer. It has been described to be involved in the reversal of NK cell exhaustion, to promote anti-tumour immunity	(Spolski *et al*. 2010; Stolfi *et al*. 2011; Kelm *et al*. 2016; Seo *et al*. 2017)
IL-22	Cytokine. Important for defence at mucosal membranes. Produced by immune cells, but acts on non-immune cells such as epithelial cells	Is known to activate STAT3 and promote cancer cell survival in CRC and HCC	(Jiang *et al*. 2011; Jiang *et al*. 2013; Rutz *et al*. 2013; Hernandez *et al*. 2018)
IL-27	Cytokine that is mostly produced by APCs. Can induce T cell differentiation and is a regulator of IL-10 production in CD4 T cells. Can induce a pro- or anti-inflammatory response depending on context	Generally accepted as having an anti-tumorigenic effect, however there is some evidence that is can also have a pro-tumorigenic effect depending on context	(Pflanz *et al*. 2002; Lucas *et al*. 2003; Hisada *et al*. 2004; Yoshimoto *et al*. 2008; Murugaiyan *et al*. 2010; Hunter and Kastelein 2012; Diakowska *et al*. 2013; Kachroo *et al*. 2013; Murugaiyan and Saha 2013; Lu *et al*. 2014; Fabbi *et al*. 2017)
IP-10 (CXCL10)	Chemokine. Binds to the CXCR3 receptor. Secreted in response to IFN-γ. Acts as an immune cell chemotactic, including T cell endothelial adhesion	Higher serum levels correspond with reduced survival and liver metastasis in CRC	(Liu *et al*. 2011; Toiyama *et al*. 2012)

MMP-1	Degrades extra-cellular matrix interstitial collagens; types I-III. Can interact with CD49b	High intra-tumour levels are associated with poor prognosis in CRC, and is linked to metastasis	(Momose *et al*. 1996; Shiozawa *et al*. 2000; Sunami *et al*. 2000; Bendardaf *et al*. 2007; Page-McCaw *et al*. 2009; Said *et al*. 2014)
MMP-2	Degrades type IV collagen in basement membranes, which are important for tissue structural integrity	Plays a role in the motility of CRC cells. Higher serum levels are found in CRC patients than healthy controls, and correlates with lymph node metastasis	(Langenskiöld *et al*. 2005; Dragutinović *et al*. 2011; Kryczka *et al*. 2012; Nelson and Guyer 2012; Said *et al*. 2014)
MMP-3	Degrades fibronectin, laminin, proteoglycans, and collagens (type III, IV, IX, X). Is known to activate other MMPs	Involved in tumour metastasis in CRC, melanoma, and breast cancer. Tumour expression is indicative of poor survival in multiple cancer types	(Ogata *et al*. 1992; Sternlicht *et al.* 1999; Mendes *et al.* 2005; Mehner *et al.* 2015; Shoshan *et al*. 2016)
MMP-7	Degrades peptidoglycan, elastin, fibronectin, and casein. Can activate defensins in lung and gut epithelial cells	Involved in tumour growth and metastasis. Expression is regulated by β-catenin in CRC, and high levels are associated with poor survival	(Wilson *et al*. 1997; Adachi *et al*. 1999; Brabletz *et al*. 1999; Burke 2004; Maurel *et al*. 2007; Said *et al*. 2014)
MMP-8	Degrades collagens type I-III. Highly expressed by neutrophils, and can drive facilitate their migration	Serum MMP-8 is associated with poor survival and systemic inflammation in CRC patients	(Lin *et al*. 2008; Väyrynen *et al*. 2012; Böckelman *et al*. 2018; Sirniö *et al*. 2018)

MMP-9	Degrades collages (types IV and V). Known roles in angiogenesis, and neutrophil migration	Linked to tumour metastasis and angiogenesis. Expression within the tumour and in the serum negatively correlates with survival in CRC. Inhibition positively influences the anti-tumour immune response in mouse models of breast cancer	(Ardi *et al*. 2007; Bradley *et al*. 2012; Kostova *et al*. 2012; Deryugina *et al*. 2014; Juric *et al*. 2018)
MMP-10	Degrades proteoglycan, fibronectin, laminin, elastin, gelatin, and collagens. Is expressed by macrophages, and can induce their activation	Drives tumour progression, metastasis, and angiogenesis. Serum expression in CRC patients is associated with poor outcomes and survival	(Zhang *et al*. 2014; Burgess *et al*. 2016; Klupp *et al*. 2016)
MMP-12	Degrades elastin, both soluble and insoluble. Involved in mediating anti-viral immunity, and the acute inflammatory response through cleavage of multiple chemokines	Serum expression associated with negative prognosis in CRC	(Dean *et al*. 2008; Marchant *et al*. 2014; Klupp *et al*. 2016)
MMP-13	Mainly cleaves type II collagen but can also cleave type I and III. Involved in cartilage degradation	Promotes tumour angiogenesis. Tumour levels correlate with poor prognosis in breast cancer, and CRC patients, and is predictive of liver metastasis	(Fosang *et al*. 1996; Zhang *et al*. 2008; Huang *et al*. 2010; Yamada *et al*. 2010; Kudo *et al*. 2012)

PIGF-1	Member of the VEGF family of growth factors, involved in angiogenesis and the promotion of inflammation	Increased expression and serum levels are associated with poor outcomes and reduced survival in CRC	(Athanassiades and Lala 1998; Wei *et al*. 2005; Wei *et al*. 2009; Kim *et al*. 2012)
RANTES (CCL5)	Chemotactic cytokine/chemokine recruiting multiple immune cell types, including T cells, and eosinophils	Increases tumour-mediated killing of anti-tumour CD8 T cells in CRC and gastric cancer. Neutralisation in mice can reduce tumour growth in CRC	(Sugasawa *et al*. 2008; Cambien *et al*. 2011; Chang *et al*. 2012; Aldinucci and Colombatti 2014; Zhang *et al*. 2018)
SDF1- α (CXCL12)	Chemokine. Induces chemotaxis in lymphocytes and monocytes. Also contributes to angiogenesis, and cell migration, proliferation, and survival	Expression of this ligand and its receptor are implicated in cancer metastasis, invasion, and angiogenesis. It is of prognostic significance in CRC	(Bleul *et al*. 1996; Brand *et al*. 2005; Zheng *et al*. 2007; Gelmini *et al*. 2008; Yoshitake *et al*. 2008; Akishima-Fukasawa *et al*. 2009; Chu *et al*. 2009)
Tenascin-C	An extra-cellular matrix protein. Mediates cell adhesion, and tissue remodelling. Is expressed during wound healing, and at sites of inflammation. It can mediate the inflammatory response, for example by activating TLR4	Increased in solid tumours. Associated with advanced stage, reduced survival, and metastasis in many tumour types	(Leins *et al*. 2003; Midwood *et al*. 2004; Midwood *et al*. 2009; Midwood *et al*. 2011; Oskarsson *et al*. 2011; Takahashi *et al*. 2013; Midwood *et al*. 2016)

TIM-3 (HAVCR2)	Type-I transmembrane protein regarded as an immune checkpoint. Inhibits Th1 and T cell responses. Soluble form can be produced from the membrane-bound form by ADAM-10 and ADAM-17	TIM-3 is increased in, and impedes upon anti-tumour immunity in multiple cancer types	(Fourcade *et al*. 2010; Clayton *et al*. 2015; Xu *et al*. 2015; Anderson *et al*. 2016; Ocaña-Guzman *et al*. 2016; Das *et al*. 2017; Gamerith *et al*. 2018)
TIMP-1	Inhibitor of MMPs. Involved in tissue remodelling, wound healing, and pregnancy. Can induce cell proliferation and prevent apoptosis	Can prevent cancer cell apoptosis and is implicated in cancer progression and metastasis. Increased serum levels in CRC and breast cancer patients, which also correlates with poor prognosis	(Holten-Andersen *et al.* 1999; Liu *et al.* 2003; Kopitz *et al.* 2007; Wu *et al*. 2008; Gong *et al.* 2013; Niewiarowska *et al.* 2014; Song *et al.* 2016; Böckelman *et al.* 2018; Meng *et al.* 2018)
VEGF-A	Growth factor. Acts on endothelial cells to induce angiogenesis, form new vessels, and increase vascular permeability	Associated with cancer angiogenesis, and metastasis in a number of tumour types. Impedes the anti-tumour immune response by inhibiting T cell development, increasing immune-checkpoint expression, and promoting T_{reg} proliferation	(George *et al*. 2001; Ohm 2003; Carmeliet 2005; Terme *et al*. 2013; Voron *et al*. 2015; Yang *et al*. 2018)

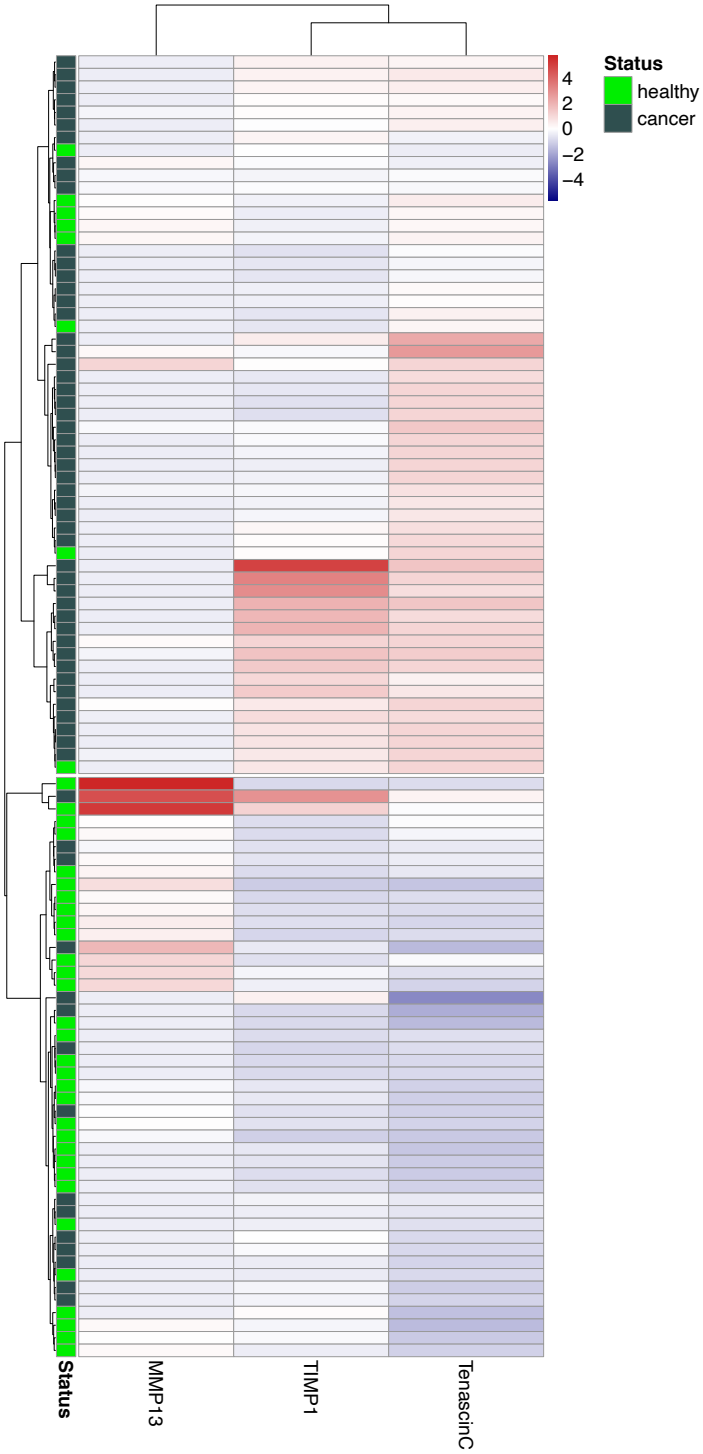
4-1BBL (CD137L)	Ligand for transmembrane receptor 41BB. The ligand is mainly on antigen presenting cells but can be expressed on T cells. It enhances the effector functions of both CD4 and CD8 T cells, but can also limit T cell activation under tolerogenic conditions	Targeting of the 4-1BB-4-1BBL pathway is of current interest in cancer immunotherapy in order to improve anti-tumour immunity. Soluble 4-1BBL has been described as increased in the serum of patients with haematological malignancy	(Salih *et al*. 2000; Cannons *et al.* 2001; Salih *et al*. 2001; Cheuk *et al.* 2004; Eun *et al.* 2015; Segal *et al.* 2017b; Zhou *et al.* 2018)

Supplementary Table 2 Overview of the plasma proteins measured by Luminex, their biological function, and their known associations with cancer and/or tumour immunity.

(D) NHS Wales Immunological/Serological Accepted Normal Ranges

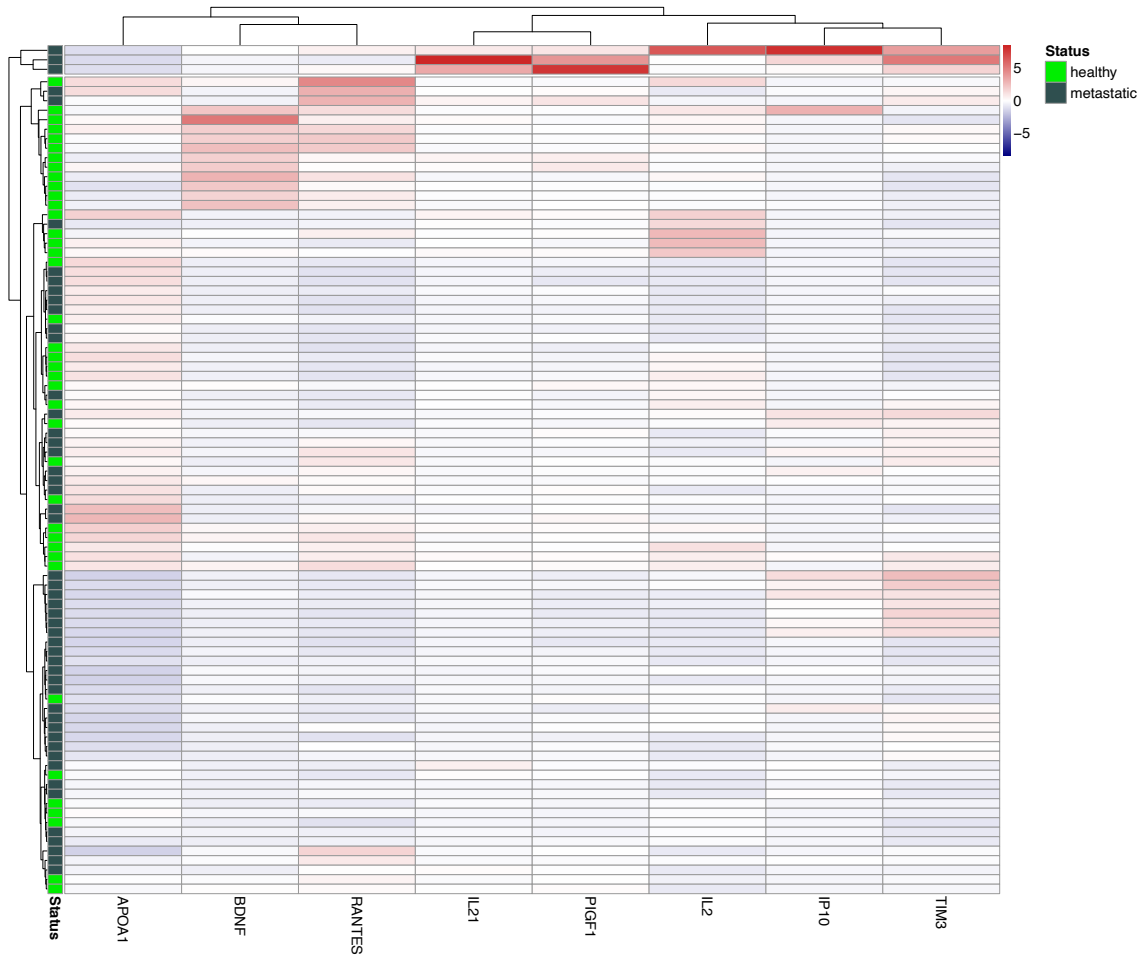
Parameter	Female	Male
ALT (U/L)	<50	<59
Basophils (x10^9/L)	0.0-0.1	0.0-0.1
Bilirubin (µmol/L)	<21	<21
Eosinophils (x10^9/L)	0.0-0.4	0.0-0.4
Glucose (mmol/L)	3.0-7.7	3.0-7.7
Haematocrit (L/L)	0.37-0.47	0.40-0.52
Haemoglobin (g/dL)	115-165	130-180
Lymphocytes (x10^9/L)	1.0-4.5	1.0-4.5
MCH (pg)	27.0-33.0	27.0-33.0
MCV (fL)	80-100	80-100
Monocytes (x10^9/L)	0.2-0.8	0.2-0.8
Neutrophils (x10^9/L)	1.7-7.5	1.7-7.5
Platelets (x10^9/L)	150-400	150-400
RBCs (x10^12/L)	3.80-5.50	4.50-6.00
WBCs (x10^9/L)	4.0-11.0	4.0-11.0

Supplementary Table 3 Accepted healthy ranges of serological and immunological measurements taken during TaCTiCC.



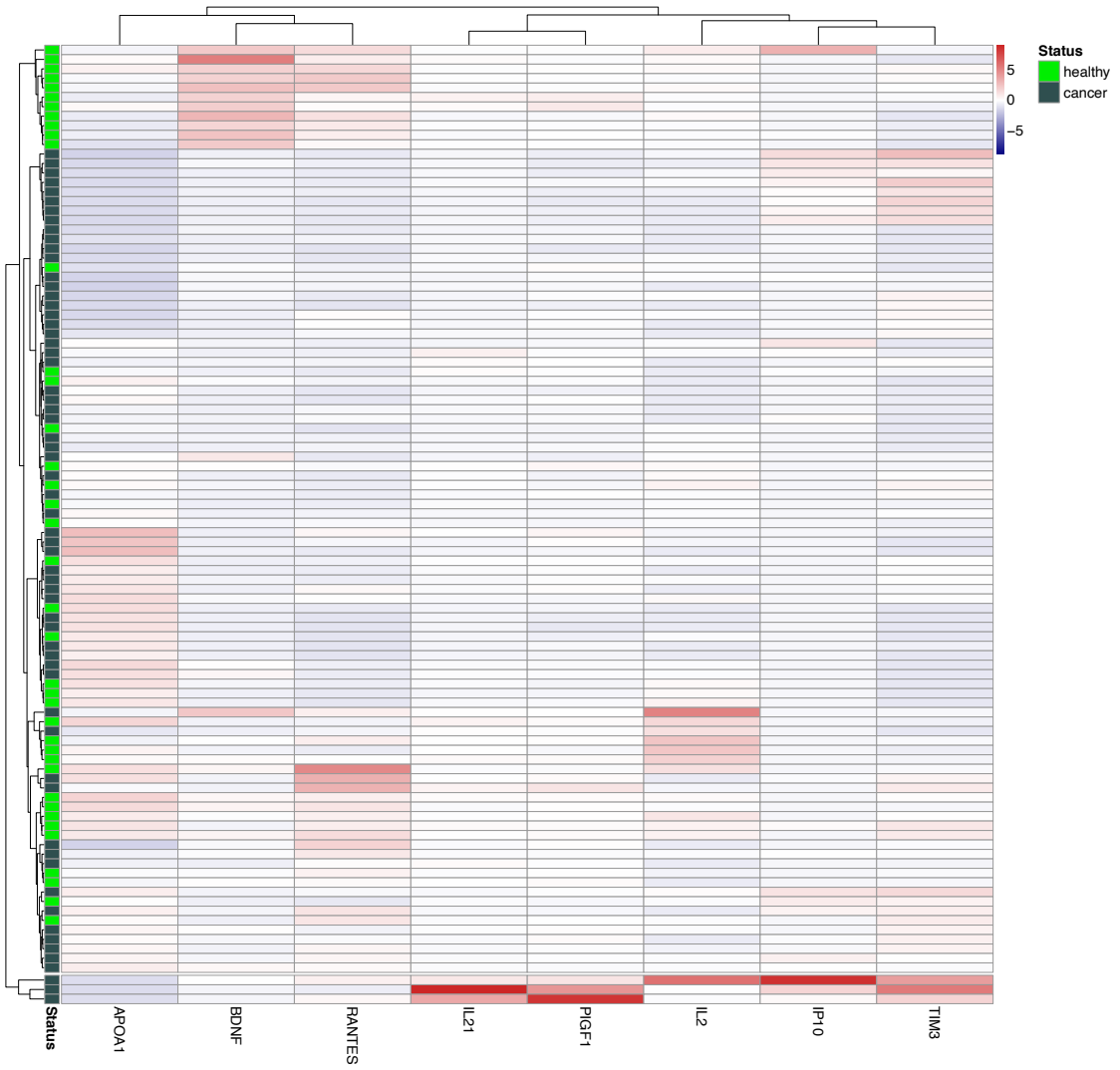
Supplementary Figure 6 A refined panel of plasma matrix proteins MMP-13, Tenascin-C and TIMP-1 can cluster CRC patients and controls.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of MMP-13, Tenascin-C and TIMP-1 for cancer patients with metastatic and non-metastatic disease (n=64) and controls (n=39). These analytes were able to separate 77% (49/64) of CRC patients and 79% (31/39) of controls into two distinct clusters.



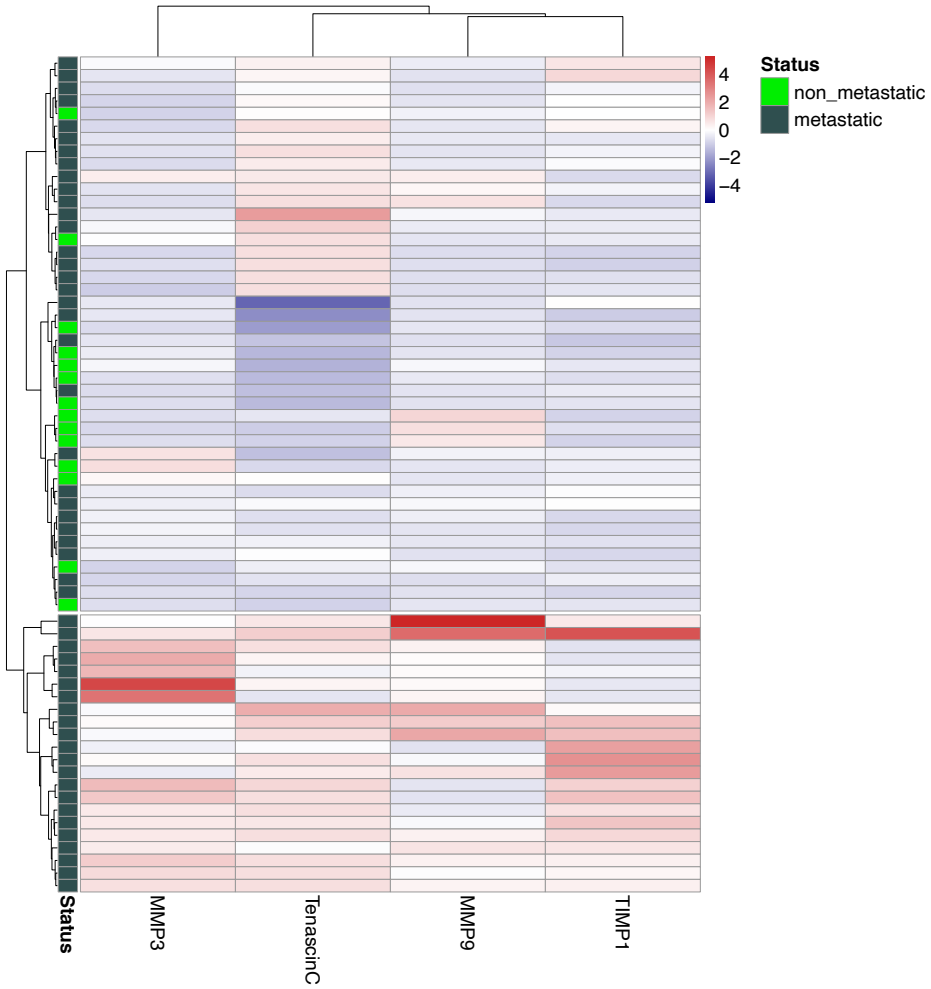
Supplementary Figure 7 Circulating levels of immune proteins and growth factors do not clearly cluster mCRC patients and controls.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of APOA1, BDNF, IL-2, IL-21, IP-10, RANTES, TIM3, and PIGF-1 for mCRC patients (n=50) and non-cancer controls (n=39). These analytes showed no clear ability to distinguish between mCRC and non-cancer.



Supplementary Figure 8 Circulating levels of immune proteins and growth factors do not clearly cluster CRC patients and controls.

Hierarchical clustering was performed in R using Ward's Method using the plasma levels of APOA1, BDNF, IL-2, IL-21, IP-10, PIGF-1, RANTES, TIM-3 for mixed-stage cancer patients (n=62) and controls (n=39). No clear expression pattern was demonstrated between the groups.



Supplementary Figure 9 Circulating levels of plasma matrix proteins are able to specifically cluster a sub-population of mCRC patients from non-metastatic patients.

Hierarchical clustering was performed in R using Ward's method. The pattern of plasma levels of MMP-3, MMP-9, Tenascin-C and TIMP-1 were compared in mCRC patients (n=52) and non-metastatic CRC patients (n=14). This was able to cluster 58% (30/52) of metastatic, and 100% (14/14) of non-metastatic into one cluster, and 42% (22/52) of metastatic patients into a separate, distinct cluster.

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