The Altered Immune Microenvironment of Human Liver in Metastatic Disease

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and

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Declaration of original authorship

I Dr. Fiona Hand, certify that this thesis which I now submit for assessment for the award of Doctor of Medicine does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference in made in the text.

I, carried out all laboratory experimental work under the supervision of Cathal Harmon in the School of Biochemistry and Immunology, Trinity College Dublin and Dr. Louise Elliott in the Centre for Colorectal Disease, St. Vincent's University Hospital, Dublin. Statistical analysis was performed by Dr. Fiona Hand and overseen by Dr. Cuan Harrington.

Dr Michael Durand helped with data entry for the retrospective cohort prior to analysis.

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Ethics statement

This study was approved by the Research Ethics Committee of St. Vincent's Hospital, Dublin. Informed written consent was obtained from all study participants who allowed their liver to be sampled.

Abbreviations

Acronym	Name
5-FU	5-fluorouracil
ANOVA	Analysis of variance
APC	Antigen presenting cell
BCA	Bicinchoninic Acid
CAPOX	Capecitabine and oxaliplatin
CCL	Chemokine (c-c motif) ligand
CCR	Chemokine (c-c motif) receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CM	Conditioned media
CRC	Colorectal cancer
CRLM	Colorectal liver metastases
CRP	C-reactive protein
CXCL	Chemokine (c-x-c motif) ligand
CXCR	Chemokine (c-x-c motif) receptor
DAMP	Damage-associated molecular pattern molecules
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EMT	Epithelial mesenchymal transition
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
FDA	Food and Drug Administration
FOLFOX	5-flourouracil, leucovorin, oxaliplatin, irinotecan
G-CSF	Granulocyte- colony stimulating factor
GMCSF	Granulocyte macrophage- colony stimulating factor
H&E	Hematoxylin & eosin
HNPCC	Hereditary non-polyposis colorectal cancer
HSC	Hepatic stellate cells
IFN	Interferon
IL	Interleukin
IMV	Inferior mesenteric vein
IP10	Interferon gamma-induced protein 10
JPS	Juvenile polyposis syndrome
KC	Kupffer cell
LIF	Leukemic inhibitory factor
LSEC	Liver sinusoidal endothelial cells
MAMP	Microbial associated molecular patterns
MAP	MUTYH-associated polyposis
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility class
MMP	Matrix metalloproteinase
MSI	Microsatellite instability

MSI-H	Microsatellite instability-high
MSI-L	Microsatellite instability-low
MSS	Microsatellite stable
NIMIS	National integrated medical information system
NK	Natural killer
NKT	Natural killer T
NLR	Neutrophil lymphocyte ratio
PD1	Programmed cell death protein 1
PDGF	Platelet derived growth factor
pg	picogram
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PJS	Peutz-jegher's syndrome [? Check]
PMN	Polymorphonuclear [leukocytes]
RFA	Radiofrequency ablation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SIRFLOX	SIRT and FOLFOX
SIRT	Selective internal radiation therapy
SMV	Superior mesenteric vein
TACE	Transarterial chemoembolization
TGF-BETA	Transforming growth factor beta
TLR	Toll like receptor
Th	T helper [cells]

TME	Tumour microenvironment
TNF	Tumour necrosis factor
TNM	Tumour node metastasis
UICC	Union for international cancer control
VEGF	Vascular endothelial growth factor

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For Freya

Thesis outputs

Awards

2nd prize Irish Society of Gastroenterology Winter Meeting, Dublin. November 2015

Publications

Depleted polymorphonuclear lymphocytes in human metastatic liver reflect an altered immune microenvironment associated with recurrent metastasis

Hand F, Harmon C, Elliott LA, Caiazza F, Lavelle A, Maguire D, Hoti E, Nolan N, Geoghegan JG, Ryan EJ, O'Farrelly C. Cancer Immunology Immunotherapy 2018 Jul;67(7):1041-1052.

Lactate-mediated acidification of tumor microenvironment induces apoptosis of liver-resident NK cells in colorectal liver metastasis

Harmon C, Robinson MW, Hand F, Almulali D, Mentor K, Houlihan DD, Hoti E, Lynch L, Geoghegan J, O'Farrelly C. Cancer Immunology Research 2019 Feb;7(2):335-346.

Chemotherapy and repeat resection abrogate the prognostic value of neutrophil lymphocyte ratio in colorectal liver metastases

Hand F, Ryan EJ, Harrington C, Durand M, Maguire D, O'Farrelly C, Hoti E, Geoghegan JG. *Accepted HPB (Oxford), September 2019*

Oral presentations

Immunoregulatory potential of the hepatic metastatic microenvironment

Hand F, Geoghegan J, Ryan EJ, O'Farrelly C; Plenary Session, Sir Peter Freyer Surgical Symposium, University College Galway, Co. Galway. September 2015

Metastatic microenvironment of human liver, characterised by increased levels of IL6, VEGF and GMCSF blocks interferon γ production by natural killer cells

Hand F, Elliott L, Harmon C, Caiazza F, Nolan N, Geoghegan J, Ryan EJ, O'Farrelly C; Irish Society of Gastroenterology Winter Meeting, Dublin. November 2015

Microenvironment of metastatic liver: rich soil for recurrent disease

Hand F, Elliott L, Harmon C, Caiazza F, Nolan N, Geoghegan J, Ryan EJ, O'Farrelly C; Moynihan Prize Session, International Surgical Congress of the Association of Surgeons of Great Britain and Ireland, Belfast, Northern Ireland. May 2016

The metastatic microenvironment of human liver characterized by increased levels of inflammatory cytokines promotes cancer cell chemotaxis

Hand F, Elliot L, Harmon C, Caiazza F, Nolan N, Geoghegan J, Ryan EJ, O'Farrelly C; Americas Hepato-Pancreato Biliary Association Annual Meeting, Miami, Florida, USA. April 2017

Poster Presentations

Metastatic liver microenvironment: relevance to hepatic NK cells

Hand F, Harmon C, Whelan S, Geoghegan J, Ryan EJ, O'Farrelly C; 15th Meeting of the Society for Natural Immunity, Quebec, Canada. May 2015

Hepatic microenvironment: the tip towards metastasis

Hand F, Elliott L, Harmon C, Caiazza F, Nolan N, Geoghegan J, Ryan EJ, O'Farrelly C; Society of Surgical Oncology 69th Annual Symposium, Boston, Massacheusetts, USA. March 2016.

Abstract

Globally, metastatic colon cancer is a significant disease with poor outcomes. Liver metastases are the leading cause of death and, despite maximal medical management, recurrent metastases are seen in up to 70% of patients with colon cancer. Surgical resection remains the only curative treatment option for these patients, despite major advances in immunotherapies for other malignant conditions. The complex immunological micro-environment of the liver and its manipulation during the development and growth of metastases presents a unique challenge to the development of effective immunotherapy for this cohort and this is the focus of this thesis.

The Department of Hepatobiliary and Liver Transplant Surgery at St. Vincent's University Hospital is the National Centre for Surgical Liver Disease. In this regard, patients are referred to St. Vincent's from the other 32 hospitals across Ireland for consideration of liver surgery. Between 1st January 2005 and 31st December 2014, 504 consecutive patients underwent hepatic metastatectomy at the Centre, 378 of whom returned to their referring hospitals for adjuvant therapy following surgery. Each patient underwent preoperative staging, comprising computed tomography of the chest, abdomen, and pelvis and primovist-enhanced magnetic resonance imaging of the liver. All patients were discussed at a weekly multidisciplinary team conference attended by oncologists, radiologists, pathologists and six hepatobiliary surgeons, where a consensus decision on resectability and sequence of treatment was made. On admission, prior to surgical resection, all patients had a routine full blood count taken where total blood leukocyte levels, including neutrophils and lymphocytes were analysed. Survival data was sought from each patients primary care physician. Elevated neutrophil counts (>8) and neutrophil lymphocyte ratio (NLR) > 5 in patients' blood preoperatively was

negatively associated with overall survival (p=0.018, p=0.027 respectively). However, in those who had received neoadjuvant chemotherapy, no association between elevated blood NLR and overall survival was demonstrated (p=0.93). Similarly, in those undergoing repeat resection, neither serum neutrophil count >8 (p=0.42) nor neutrophil lymphocyte ratio >5 (p=0.81) correlated to postoperative survival.

The microenvironment generated by any tumour is a dynamic interface between tumour cells and host stromal cells, fibroblasts, endothelial cells immune populations and their secretomes. Cytokines and chemokines within this microenvironment play key roles in the regulation of immune cell infiltration of the tumour and their activity. Immune cells recruited to malignant sites actively secrete cytokines and chemokines which attract and dictate the activity of tumour cells and other immune cells within the metastatic niche. Healthy human liver is replete with immune cells poised to provide effective anti-tumour surveillance. We hypothesise that for sustained tumour growth within the liver, its inherent immune surveillance mechanism is compromised. We sought to characterise the altered immune microenvironment of metastatic liver with a view to identifying biomarkers that may be associated with patient outcome, and potential targets for the development of immunotherapy.

Liver tissue was obtained from 25 patients from the above cohort undergoing hepatectomy for CRLM at SVUH between November 2014 and March 2015. On follow up at 18 months, 13 of these 25 went on to develop intrahepatic recurrence. Normal liver tissue was obtained from 12 donor livers at the time of organ retrieval prior to liver transplantation. Histological analysis revealed that periportal polymorphonuclear leukocytes were depleted and lymphocytes were increased in metastatic liver when

compared to donor liver tissue. Interestingly, liver NLRs were lower in liver tissue distal to the metastasis when compared to donor. Patients with the fewest PMNs in liver tissue distal to their tumour had a shorter time to intrahepatic recurrence ($P<0.001$). This is the inverse of what we found on analysis of our cohort's peripheral blood NLR, where elevated neutrophils were negatively associated with patient survival, emphasising the unique immunological environment of the liver .

Conditioned media from three areas of metastatic liver were analysed by protein array and compared to donor liver samples. A number of cytokines, chemokines and growth factors were found in high concentrations in all samples of liver tissue emphasising the immunological complexity of the hepatic microenvironment. Twelve cytokines were found to be differentially expressed in metastatic liver compared to donor liver tissue. Multiplex analysis of each of these differentially expressed cytokines (IL-6, CXCL1, CXCL5, G-CSF, GM-CSF, VEGF, LIF, and CCL3) confirmed higher expression in CRLM-bearing liver compared to donor liver tissue. Even in liver distal from the tumour (often described as 'normal' in other studies), levels of GM-CSF, LIF and IP10 were significantly elevated when compared to donor liver tissue indicating that the entire organ responds to the presence of malignancy. In vitro chemotactic assays revealed that cancer cells migrated towards conditioned medium from all regions of metastatic liver but not towards donor liver CM, supporting the view that the whole organ is effected by cancer growth.

This study adds to increasing evidence that the host immune system is altered in the presence of malignant disease. In particular, the immune microenvironment of metastatic liver is manifest by a changing cytokine profile and changing immune cell concentrations

in tissue at a distance from the tumour. As a result of these alterations, metastatic liver is chemo attractive to CRC tumour cells. Following curative liver resection, recurrent metastatic disease is common and correlates with decreased neutrophil densities in metastatic hepatic parenchyma. Contrary to our findings in metastatic liver, increased neutrophil levels and high NLR in peripheral blood were associated with poorer overall outcomes, however the mechanism by which high NLR contributes to malignant progression is still poorly understood. A better understanding of these changes will be required in order to successfully manipulate the hepatic immune response following the establishment of metastasis.

Chapter 1: Introduction

1.1 Epidemiology of colorectal cancer

Colorectal cancer, second only to lung for its contribution to cancer mortality nationally (NCRI, 2018) continues to pose a significant health problem. It represents Ireland's most prevalent gastrointestinal malignancy, with an annual incidence of 2,400 cases (National Screening Service, 2016). Currently, approximately 50% of patients with CRC develop liver metastases during the course of their disease (Geoghegan and Scheele, 1999), with metastases the chief determinant of life expectancy in this patient cohort (Brachet D, 2009). CRC affects men and women almost equally with a slightly higher incidence in males. While traditionally, a diagnosis associated with an older population, recently there has been an increase in the diagnosis amongst young adults (<50 years) in the United States and Australia (Edwards BK, 2010; Young JP, 2015).

Diet has been strongly associated with the increasing incidence of CRC, notably the increasing popularity of fatty foods and red or processed meat (more than 30g/day). Alcohol has also been attributed to the increased incidence of colonic malignancy (Irigaray P, 2007) . Owing to public education initiatives and screening programmes, overall rates of the disease are decreasing in the United States, New Zealand and France (Torre LA, 2016). Conversely, the incidence of colorectal malignancy in Latin America, Asia and Eastern Europe has risen significantly over the last two decades, possibly attributable to a more sedentary lifestyle, changing dietary patterns and smoking (Zhang J, 2012; Chatenoud L, 2014). Most CRCs occur sporadically however some patients have an inherited form of CRC (Labianca R, 2010) and can occur more frequently in those with inflammatory bowel disease and type 2 diabetes (Labianca R, 2010; Larsson SC, 2005). While smoking and physical inactivity (Larsson SC, 2006; Mehta M, 2014) have been linked to colonic malignancy, exercise not only correlates with improved survival

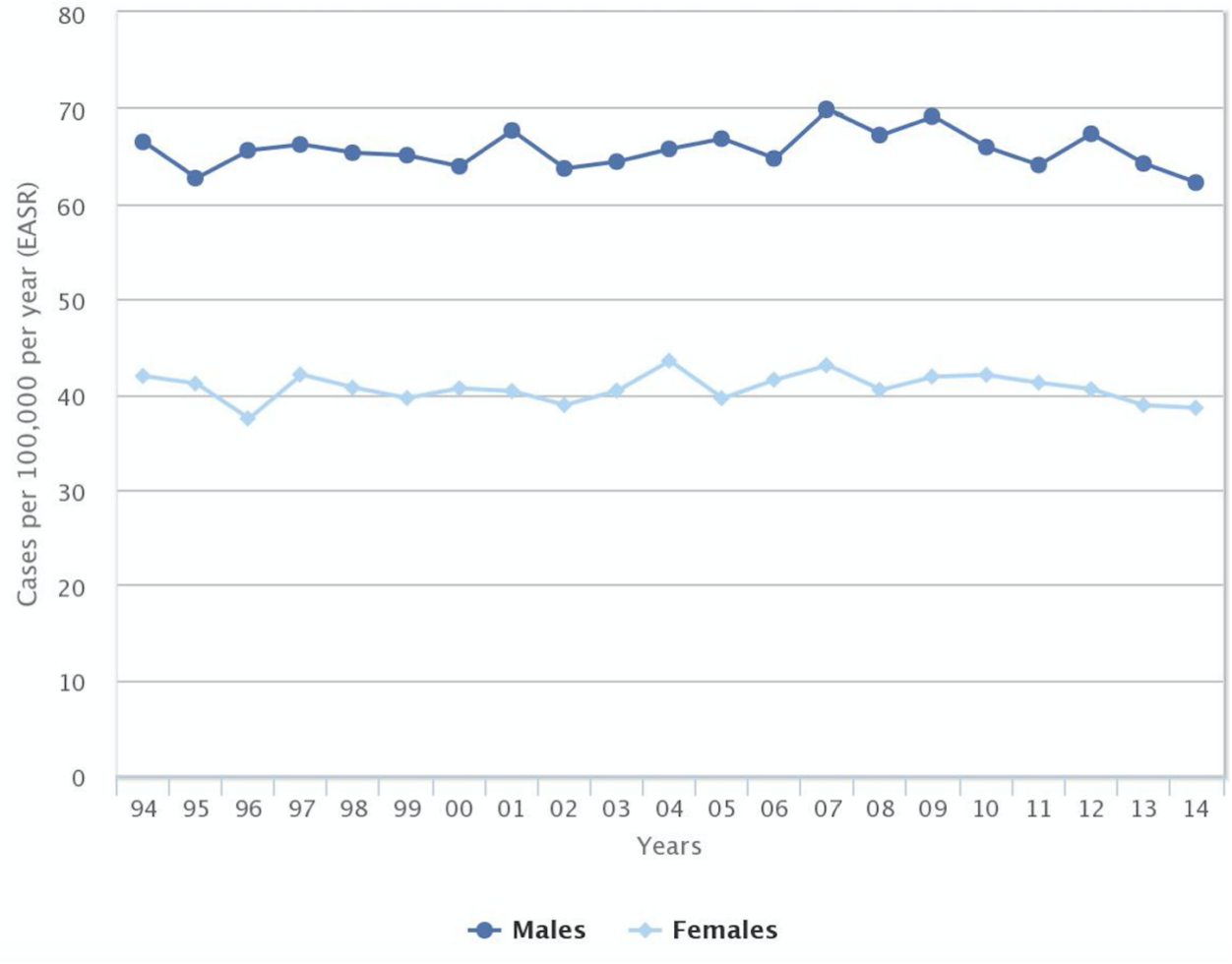


Figure 1.1 Incidence of CRC in Ireland 1994-2014, Irish national cancer registry.

(National Cancer Research Ireland, 2018)

rates in CRC, but is also associated with improved quality of life in survivors of CRC (Denlinger CS, 2011).

1.2 Colorectal carcinogenesis

Colorectal cancer is a heterogenous disease arising from a combination of both environmental and genetic factors, with approximately 80 genetic mutations found in each tumour. Normal colonic epithelium undergoes a series of progressive changes to form adenomas which advance to invasive malignancy. This adenoma to carcinoma sequence arises from activation of proto-oncogenes and blockade of tumour suppressor genes and manifests as histological changes within the colonic epithelium (Figure 1.2). The first genetic event in CRC; mutations in the APC gene causes the Wnt signalling pathway to become activated. In addition, KRAS proto-oncogene, mutated in up to 50% of CRC activates the MAPK pathways promoting cell survival and inhibiting apoptosis (Downward J, 2003). Almost 30% of colorectal tumours exhibit mutations in the *TGFBR2* gene causing inactivation of the TGFβ pathway. This pathway inhibits intestinal epithelial cells growth and induces apoptosis (Zhang W, 2014). A further 30% of colorectal tumours have a mutation in the *PI3KCA* gene, which activates PI3K signalling, resulting in increased cell survival (Zhang J, 2011). Microsatellite instability (MSI) resulting in a molecular phenotype associated with increased immunogenicity can also be a feature of colorectal tumours. Occurring following deficient DNA mismatch repair mechanisms, MSI leads to an increased number of genetic mutations within tumours and is considered of prognostic value (Moline J, 2013). The presence of microsatellite instability is also associated with lower rates of metastatic disease and superior patient outcomes when compared to microsatellite stable tumours (Gryfe R, 2000), emphasising its importance.

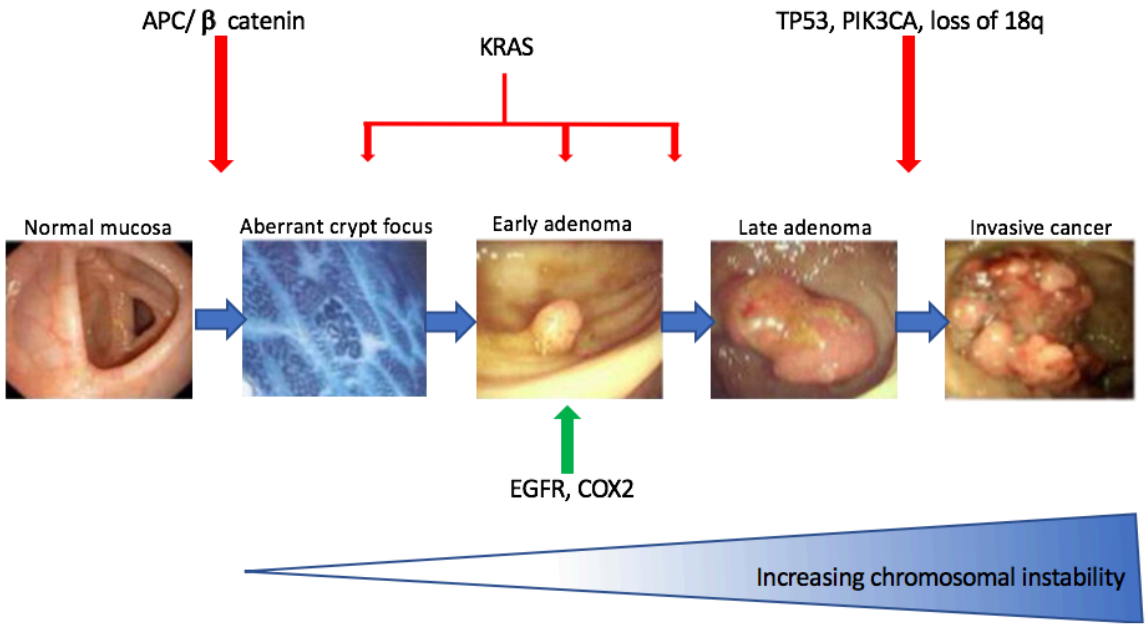


Figure 1.2 Colorectal carcinogenesis. Activation of the Wnt pathway enables adenomas to develop in colonic mucosa. For these adenomas to progress to carcinoma various genetic mutations need to occur including mutations in either KRAS or TP53, or loss of heterozygosity at chromosome 18q. The level of chromosomal instability increases as tumour progression occurs. Adapted from Pino et al (Pino MS, 2010).

1.3 Metastasis in colorectal cancer

When cells metastasise they undergo epithelial mesenchymal transition (EMT) allowing them increased migratory capacity, invasiveness and resistance to apoptosis. In colorectal cancer, epithelial mesenchymal transition is seen as tumour buds, either single cells or small clusters of de-differentiated tumour cells at the invasive margin (Kalluri R, 2009). Tumour budding is predictive of lymph node metastasis, vascular and lymphatic invasion, solid organ metastasis, and cancer related death (Rogers AC, 2016). For tumours that successfully metastasize, malignant cells shed from the primary tumour into the circulation where they disseminate around the body. These tumour cells are either transported in the vasculature and exit the circulation at the organ of interest (Sleeman JP, 2011) or they can enter the lymphatic system and be transported to regional lymph nodes.

Liver metastases are the most frequently encountered in colorectal cancer and are diagnosed in 50% of patients with the disease. The superior and inferior mesenteric veins (SMV, IMV) act as the main drainage route of blood from the colon. These veins bring nutrient rich blood from the colon to the liver via the portal vein, thus providing a direct link for haematogenous dissemination of malignant cells (Augestad KM, 2015). The lymph drainage of the colon resides in the submucosal layer of the bowel. As the primary colon tumour grows and invades the deep layers of bowel wall, the muscularis propria is breached. This enables the proximal spread of malignant cells to the epicolic, paracolic, lymph nodes of the SMV and IMV and finally para-aortic lymph nodes.

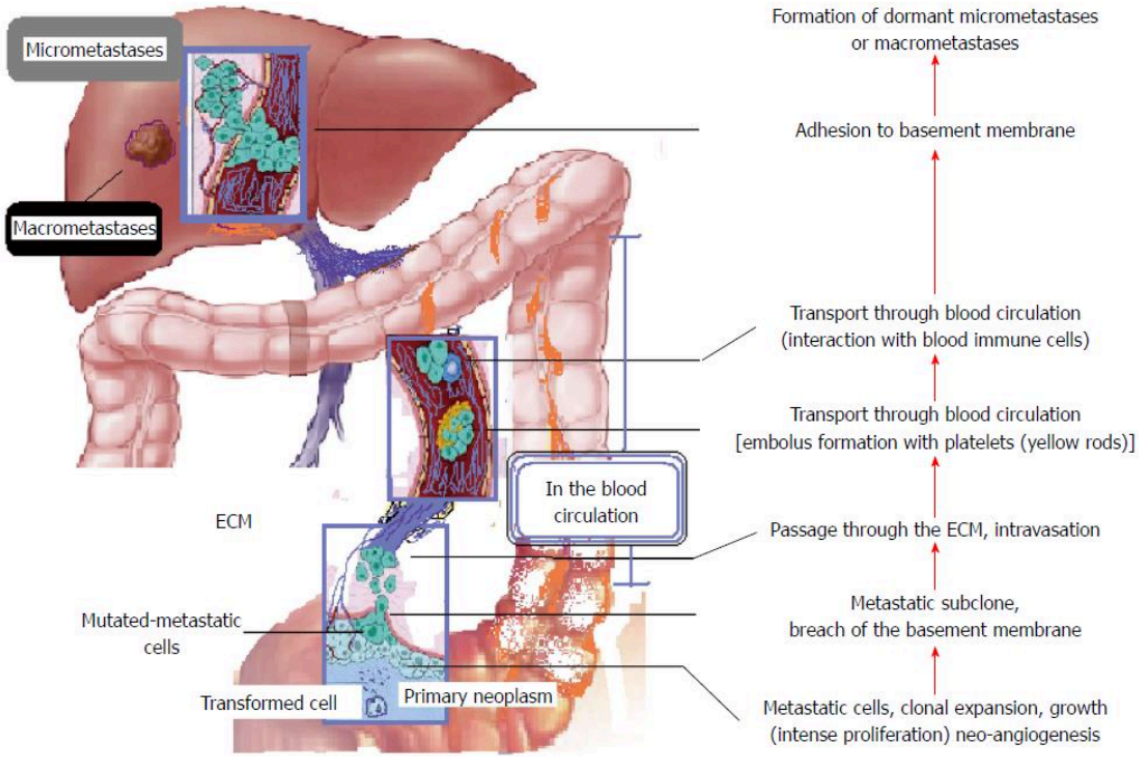


Figure 1.3. Stepwise development of colorectal liver metastases from invasive colon cancer. Metastatic cells develop and proliferate before breaching the basement membrane. Following passage through the extracellular matrix (ECM), metastatic cells gain access to the blood stream via the superior and inferior mesenteric veins and the portal vein. On presentation to the liver, they adhere to the basement membrane to form either dormant micro metastases or macro metastases. Adapted from Pachos et al. (Paschos KA, 2014)

1.4 Tumour Immunology

In the healthy patient, a competent immune system is capable of eliminating malignant cells, halting tumour progression. For tumours to proliferate and survive, they must develop mechanisms to evade the host immune response (Hanahan D, 2000). This can be achieved by the tumours ability to create a pro-malignant niche, rich in immunosuppressive cytokines to manipulate immune cells. Tumours have also been shown to exhibit powerful suppressor activity by selectively enriching the local microenvironment with powerful suppressor cells. T_{reg} cells and myeloid-derived suppressor cells (MDSCs) are two examples which have been heavily implicated in the progression of cancer by suppressing the immune system, contributing to the tumour microenvironment and encouraging the differentiation of more suppressor cells (Gabrilovich DI, 2009)

The type or quality of a tumour can also determine the type of immune response generated. Tumours developed in immunocompromised mice lacking T, B, NKT lymphocytes are more easily rejected when transplanted into naive wildtype (WT) mice. In contrast, all sarcomas derived from immunocompetent wildtype mice grow progressively when transplanted into naive syngeneic wild-type hosts (Shankaran V, 2001). Therefore, tumours formed in the absence of an intact immune system are more immunogenic than tumours that grow in immunocompetent hosts. This new data led to the formulation of the "cancer immunoediting" hypothesis; which emphasized the ability of the immune system to either inhibit or potentially promote the growth and immunogenicity of tumours.

The immunoediting process of how tumours develop and are monitored by the immune system is divided into three main phases: elimination, equilibrium and escape (Dunn GP,

2002; Dunn GP, 2004). Elimination involves molecules and cells of both innate and adaptive immunity working together to detect the presence of a developing tumour and destroy it long before it is established. Tumour cells may sometimes not be completely eliminated and instead enter into an equilibrium phase where the immune system controls tumour cell expansion and growth. In this equilibrium phase, tumour cells can become functionally dormant and remain clinically unapparent for the life of the host. Therefore, equilibrium is a second potential stable endpoint of cancer immunoediting. Escape occurs where change either occurs in the tumour cell population due to an active immunoediting process or in the host immune system (resulting from increases in cancer-induced immunosuppression or immune system breakdown due to the natural aging process). As a result, restrictions on the growth of the tumour cell population may break down and allow them to grow in an immunologically unrestricted manner until presenting as a clinically apparent disease.

Immunological scores analysing density and type of tumoural immune cell infiltrate are acknowledged as accurate predictors of overall survival (Galon J, 2014) with routine measurement of tumour immune infiltrates now recommended (Kirilovsky A, 2016). In fact, increased densities of $CD3^+$ and $CD8^+$ lymphocytes at the invasive margin of rectal tumours have been found to have significant predictive value in terms of disease free and overall survival (Anitei MG, 2014).

1.5 Consensus molecular subtypes of colorectal cancer

Four distinct "Consensus Molecular Subtypes" introduced by an international expert panel classify colorectal tumours according to their differing genetic profiles and immune cell infiltrates (Figure 1.3) Consensus molecular subtype 1 (CMS1) are tumours characterised by mutations in the BRAF proto-oncogene, the presence of microsatellite

instability and immune evasion pathways. Consensus molecular subtype 2 (CMS2) tumours exhibit chromosomal instability with activation of both the Wnt and MYC pathways. Mutation in the KRAS proto-oncogene is associated with consensus molecular subtype 3, with disruption of metabolic pathways also seen in this subtype. Consensus molecular subtype 4 tumours display stromal infiltration with increased levels of transforming growth factor beta (TGFβ) and angiogenesis along with mesenchymal gene expression (Guinney J, 2015).

These four distinct molecular subtypes have distinct prognoses independent of tumour stage; subtype 4 tumours are associated with shorter disease free and overall survival. Subtypes 2 and 3 however appear to be devoid of immune cell infiltration, implying alternative immune escape mechanisms are needed to sustain their growth. The importance of understanding these immune evasion mechanisms is highlighted by the fact that subtypes 2 and 3 account for 50% of all colorectal tumours. The tumour microenvironment is an important contributor to gene expression signals in tumours, in fact each consensus molecular subtype has a distinct tumour microenvironment. This is reflected by high levels of CD8+ T lymphocytes found in both subtypes 1 and 4. In addition high frequencies of KRAS mutations are found in subtype 3. These findings have implications for treatment options as monoclonal antibodies targeting EGFR (e.g. Cetuximab) are ineffective in KRAS driven tumours. The heterogeneity exhibited by colorectal tumours is a reflection of the different types and timing of genetic mutations and their interaction with host stroma throughout tumour development. Carcinogenesis is a "multi- molecule" phenomenon; an understanding of the heterogeneity present in tumour, stromal and immune components is the key to more personalised and effective treatment. Whilst certain mutations such as KRAS exhibit concordance between primary colorectal tumours and corresponding metastases in the same patient (Kim TM, 2015).

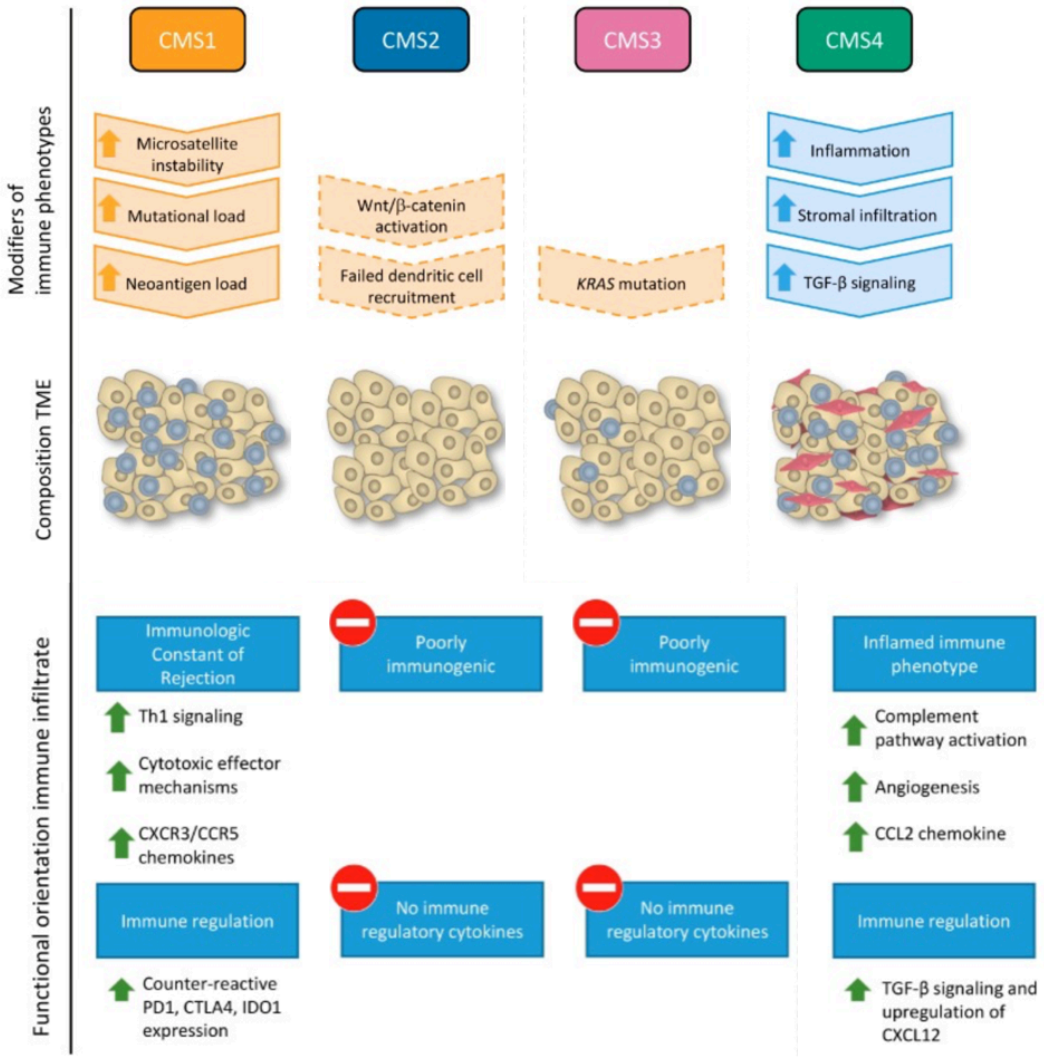


Figure 1.4 Consensus molecular subtypes of colorectal cancer. Each profile is associated with a characteristic immune cell profile. Potential genetic modifiers of each immune phenotype are highlighted in orange, environmental modifiers are highlighted in blue. Adapted from Roelands et al (Roelands J, 2017).

Other mutations including those associated with loss or gain of function differ between the primary tumour and its metastasis leading to inter tumour heterogeneity. This may reflect the acquisition of additional mutations during metastatic spread. The finding of genetic heterogeneity between primary colorectal tumours and their metastases corresponds to an altered immune cell infiltrate and tumour microenvironment. Indeed, increased levels of heterogeneity between the two correspond to poorer patient outcomes. (Sveen A, 2016).

1.6 The immune microenvironment of colorectal liver metastases

Distinct from primary liver cancer, liver metastases form in what is a healthy liver without underlying inflammation or cirrhosis. A competent hepatic immune system has the ability to successfully block the development of metastases. Hepatic NK cells can suppress the formation of colorectal liver metastases through tumour necrosis factor-related apoptosis inducing ligand (TRAIL) expression (Takeda K, 2001). This occurs via inflammasome induced IL18 expression by Kupffer cells (Dupaul-Chicoine J, 2015), demonstrating active surveillance of "non-hepatic" cells by lymphocytes in the liver to protect against metastases. Nonetheless, colorectal liver metastases are common, suggesting that hepatic immune surveillance is subverted.

To ensure metastatic success, primary tumours expand favourable immune cell populations in the liver via secretion of cytokines and chemokines, to create a pro-metastatic niche, attractive to circulating tumour cells (Hoshino A, 2015). Cytokine and chemokine expression within the metastatic tumour microenvironment play a key role in the regulation of immune cell infiltration. Neutrophils recruited to the site of tumour actively secrete cytokines and chemokines which not only encourage the recruitment of further neutrophils but also attract and dictate the activity of other immune cells within

the microenvironment (Sionov RV, 2015). In fact, two distinct cytokine signatures exist for both N1 and N2 tumour associated neutrophils (TANs). N1 TANs produce high levels of pro inflammatory cytokines IL12 and TNFα in addition to T cell and macrophage chemo attractants; CXCL10, CCL2, CCL3, CCL7 (Shaul ME, 2016). Furthermore, CXCL14, a potent chemoattractant for dendritic cells and NK cells is downregulated in N1 TANs when compared to N2 TANs. CCL17 is actively secreted by N2 TANs which in turn recruits Tregs to the tumour microenvironment. Depletion of tumour neutrophils has been shown to result in a marked reduction of Treg migration to the tumour (Mishalian I, 2014). This reinforces the concept that TANs are a potent source of chemokines and cytokines within the tumour and therefore a key component in the tumour microenvironment.

High frequencies of intratumoural TANs correlate with higher incidence of tumour recurrence and shorter disease free and overall survivals (Shen M, 2014). Furthermore, TANs have been shown to facilitate seeding of tumour cells to metastatic sites (Wculek SK, 2015) and support angiogenesis (Tazzyman S, 2013). Under certain conditions TANs demonstrate direct antitumour effects (Ishihara Y, 1998) and have been shown to abrogate the development of metastases (López-Lago MA, 2013). This concept of neutrophil 'plasticity' has been exemplified by Fridlender et al who demonstrated a pro-tumour (N2) phenotype of TANs in the presence of TGF-β. In contrast, blockade of TGF-β or type 1 interferons result in an antitumour (N1) phenotype (Fridlender ZG, 2009; Fridlender ZG, 2012). Furthermore, TANs isolated from early stage tumours are more cytotoxic to malignant cells than TANs isolated from mature established tumours (Mishalian I, 2013). This has led to the hypothesis that TANs switch from an N1 to N2 phenotype during tumour progression.

Frequencies of TILs are found to correlate between primary colorectal tumours and their liver metastases (Shibutani M, 2018), and when present in high concentrations are associated with improved prognosis (Halama N, 2011). Specifically, CD8+ T cells play a central role in antitumour immunity by directly attacking tumour cells (Kim Y, 2015). . However, constant exposure to tumour antigens can result in a state of exhaustion and lymphocyte dysfunction in essence facilitating immune escape and worse patient outcomes (Ahmadzadeh M, 2009). Programmed cell death -1 (PD-1), a cell surface receptor molecule on T cells is a marker for T cell dysfunction (Jiang Y, 2015). Its ligand, PDL-1 is expressed on tumour cells, macrophages and dendritic cells. By binding PD-1, PDL-1 communicates an inhibitory signal resulting in T cell dysfunction, anergy and apoptosis. While Shibutani *et al* failed to demonstrate any prognostic significance between the number of PD-1 TILs and clinical outcome of patients with colorectal cancer, they found patients with a high PD-1/CD 8+ T cell ratio to have a significantly shorter disease free and overall survival (Shibutani M, 2017).

1.7 The immune repertoire of healthy liver

As the host response to malignancy has emerged as a critical determinant of oncologic outcome, so too the livers response to the tumour microenvironment shapes the patient's clinical course (Kanas GP, 2012). Human liver is a key immunological organ, equipped with diverse populations of immune cells (Nemeth E, 2009). The liver is constantly presented with gut derived molecules that have the potential to instigate hepatic inflammation. Yet, healthy liver tolerates homeostatic inflammation, mounting an immunological defence when needed in response to pathogens and malignant cells (Robinson MW, 2016). Tolerance is a well-recognized phenomenon in clinical hepatology; liver transplantation is performed without HLA matching, requires less

immunosuppression postoperatively (Mazariegos GV, 1997) and has less incidences of rejection than other transplanted organs. Additionally, when the liver is used as part of a multiorgan transplant, there is a higher rate of graft acceptance (Calne RY, 1969). This has been attributed to several tolerogenic mechanisms (Bettens F, 2005; Dou L, 2018; Thomson AW, 2010)**,** as well as the unique immune cell populations resident in the liver.

Exposed to dietary antigens, pathogens and environmental antigens via the portal vein, a direct route from the gut; the liver is a key site of pathogen recognition. Thus, it requires a specialized resident immune system to protect itself from harmful pathogens and remain tolerogenic when faced with harmless dietary antigens (O'Farrelly C, 1999). Healthy human liver is replete with immune cells, including natural killer (NK) cells, T cells, natural killer T (NKT) cells (Doherty DG, 1999; Norris S, 1998), neutrophils, liver specific macrophages (Kupffer cells) (Smedsrød B, 1994) and dendritic cells (DCs) (Doherty DG, 2001).

1.8 Lymphoid liver immune cells

Between one third and one half of the liver's lymphocytes are NK cells, the highest frequency observed in any organ. Innate effectors, NK cells are able to respond to lyse target cells via the release of perforin or granzyme, they can also respond to altered expression of host major histocompatibility complex (MHC) or of self-antigens. (Vivier E, 2011). Furthermore, in response to stimulation they have the ability to produce cytokines such as IFN γ which in turn can shape and direct the immune response (Fauriat C, 2010). While liver resident NK cells have been shown to produce less IFN γ in response to stimulation, they display higher levels of inhibitory receptor NKG2A. Additionally it has been reported that liver resident NK cells may be suppressed by high IL10 levels, produced by DCs in the liver. This suggests that in the healthy hepatic

microenvironment, NK cells are maintained in a quiescent state but reserve the ability to be "switched on" if required (Krueger PD, 2011). In addition, these cells may be suppressed by high interleukin (IL-10) levels (produced by DCs) associated with the liver. Through their effects on dendritic cells (DCs), NK cells have the ability to mold the adaptive immune response. This dialogue results in DC maturation and encourages DC secretion of both cytokines and chemokines which in turn dictate downstream

T cell responses (Andrews DM, 2003) . NK cells have also been shown to selectively kill macrophages, DCs and T cells, thereby suppressing the adaptive immune response (Krueger PD, 2011). T cells account for up to 40% of the liver's lymphocytes (Doherty DG, 2000),playing a key role in cell mediated immunity. Their defining characteristic is a T cell receptor (TCR), and they are identified by CD3, a TCR co-receptor (Castro CD, 2015). Once activated, T cells undergo clonal expansion; mature CD4+T cells trigger the activation of innate immune cells and B cells. CD4+ T cells can also activate CD8+T cells which in turn lyse target cells upon recognition of their specific antigen (Zhang N, 2011). In the liver, $CD8^+$ T cells outnumber $CD4^+$ T cells two to one; a ratio which is reversed in blood (Doherty DG, 2000). Although a large number of T cells in the liver can lyse target cells, the majority of liver T cells also rapidly secrete cytokines. In its healthy state, the liver is maintained in a tolerogenic state yet despite this, up to 95% of T cells secrete inflammatory (T helper (Th) 1) cytokines like IFN-γ, TNF-α and IL-2 upon stimulation. This suggests that while the liver is in a state of equilibrium, there are mechanisms in place to ensure T cell quiescence.

NKT cells are lymphocytes sharing characteristics of both NK cells and T cells. Sharing many of the functions of both NK cells and T cells, they have the ability to secrete a variety of both Th1 and Th2 cytokines while retaining potent cytotoxic abilities (Terabe M, 2018). Like other innate lymphocytes, NKT cells are enriched in the liver compared

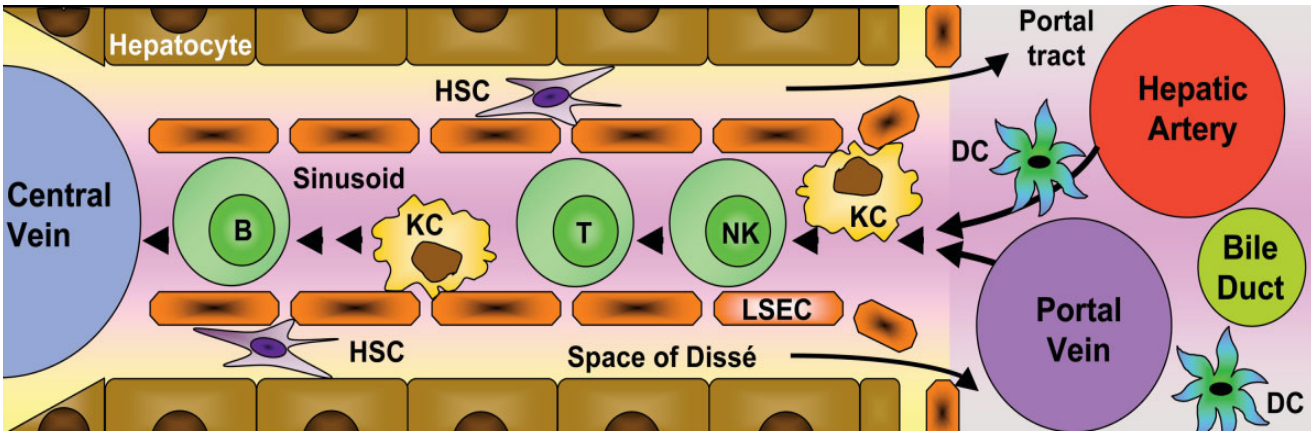


Figure 1.5: Immune cells in the liver. Blood enters the liver at the portal triads, passes through a network of liver sinusoids, and leaves the liver via the central hepatic vein. The liver sinusoids are lined by a fenestrated layer of sinusoidal endothelial cells (LSECs). The portal tracts and liver sinusoids are interspersed with Kupffer cells (KCs), dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, and T cells. The space of disse contains the hepatic stellate cells (HSCs). Adapted from (Racanelli and Rehermann, 2006).

to blood. Similarly, γδ T represent a large percentage of liver lymphocytes, and mediate liver inflammation (Ravens S, 2018) with a significant increase in the number of γδ T cells in the liver of tumour-bearing mice (Hammerich L, 2014). Elevation of γδ T cells was also found in the livers of patients with viral hepatitis infection, but not in patients with non-viral hepatitis (Tseng CT, 2001). γδ T cells may play a prominent role in innate defense against infection and malignancy (Kobayashi H, 2007). The different phenotypes displayed in hepatic γδ T cells are tailored towards anti-tumour immunity (Kenna T, 2004).

1.9 Myeloid liver immune cells

Kupffer Cells, specialized in phagocytosis are liver resident macrophages and account for 90% of tissue macrophages in the entire body. Derived from monocytes, they account for almost 35% of the non-parenchymal cells in the liver and play an important role as professional APCs with the potential to activate all lymphoid cell subsets (Bilzer M, 2006). Macrophages in response to environmental stimuli have been shown to exhibit both functional and phenotypical plasticity (Mantovani A, 2013). In healthy environments, macrophages contribute to the maintenance of homeostasis, coordinating tissue remodeling via phagocytosis. However, in response to cytokine or toll like receptor signaling, macrophages release reactive oxygen species (ROS) and instigate pathogen phagocytosis. KCs remain stationary within the vasculature of the liver adherent to LSECs and therefore constantly exposed to the contents of the hepatic vascular inflow (McCuskey RS, 2008). This has significance for their ability to capture bacteria as they arrive at the liver; KCs have unique complement receptors with the ability to bind to complement component 3b (Dixon LJ, 2013). In the absence of KCs or their receptors, bacteria have the opportunity to flourish resulting in overwhelming sepsis and death (Helmy KY, 2006).

Neutrophils account for 50-70% of circulating lymphocytes in healthy humans, playing a key role in host defense. Endowed with the ability to extravasate from the circulation and enter tissues to fight pathogens, they phagocytose and kill microorganisms by releasing cytokines such as TNFα, IL-1, and interferons along with defensins, toxic substances and reactive oxygen species (Oliveira THC, 2018). However, neutrophils also contribute to host defense in the production of both pro and anti-inflammatory cytokines (IL1α/β, IL-6, IL-4) immunoregulatory cytokines (IL-12, IFN-α/γ), colony-stimulating factors, angiogenic factors and members of the TNF superfamily (Mantovani A, 2011).

Until recently, the role of neutrophils in this communication between immune and tumour cells has been underestimated. Under certain conditions, such as overwhelming injury or malignancy or infection, expansion of a subset of neutrophils has been shown to block the expansion of T cell populations (Kalyan S, 2014). This neutrophil-like subpopulation known as myeloid derived suppressor cells (MDSCs) are actually heterogenous, characterized by a morphological mixture of granulocytic and monocytic cells, lacking the expression of cell-surface markers associated with fully differentiated monocytes, macrophages or DCs (Gabrilovich DI, 2009). In fact, granulocytic MDSCs have similar morphology and cell surface markers [details] to mature neutrophils. The only differentiating feature between mature neutrophils and granulocytic MDSCs in fact is their suppressive ability (Nagaraj S, 2007). This has led some to question whether indeed these are two distinct populations or in fact a single cell type with inherent plasticity, switching from active phagocytosis to potent immunosuppression depending on the local environment (Tcyganov E, 2018).

The differentiation of MDSCs is an area of intense research. They can add to their numbers by forcing myeloid cells recruited to the tumour microenvironment to become MDSCs, but they can also differentiate into pro-tumour subsets of other myeloid

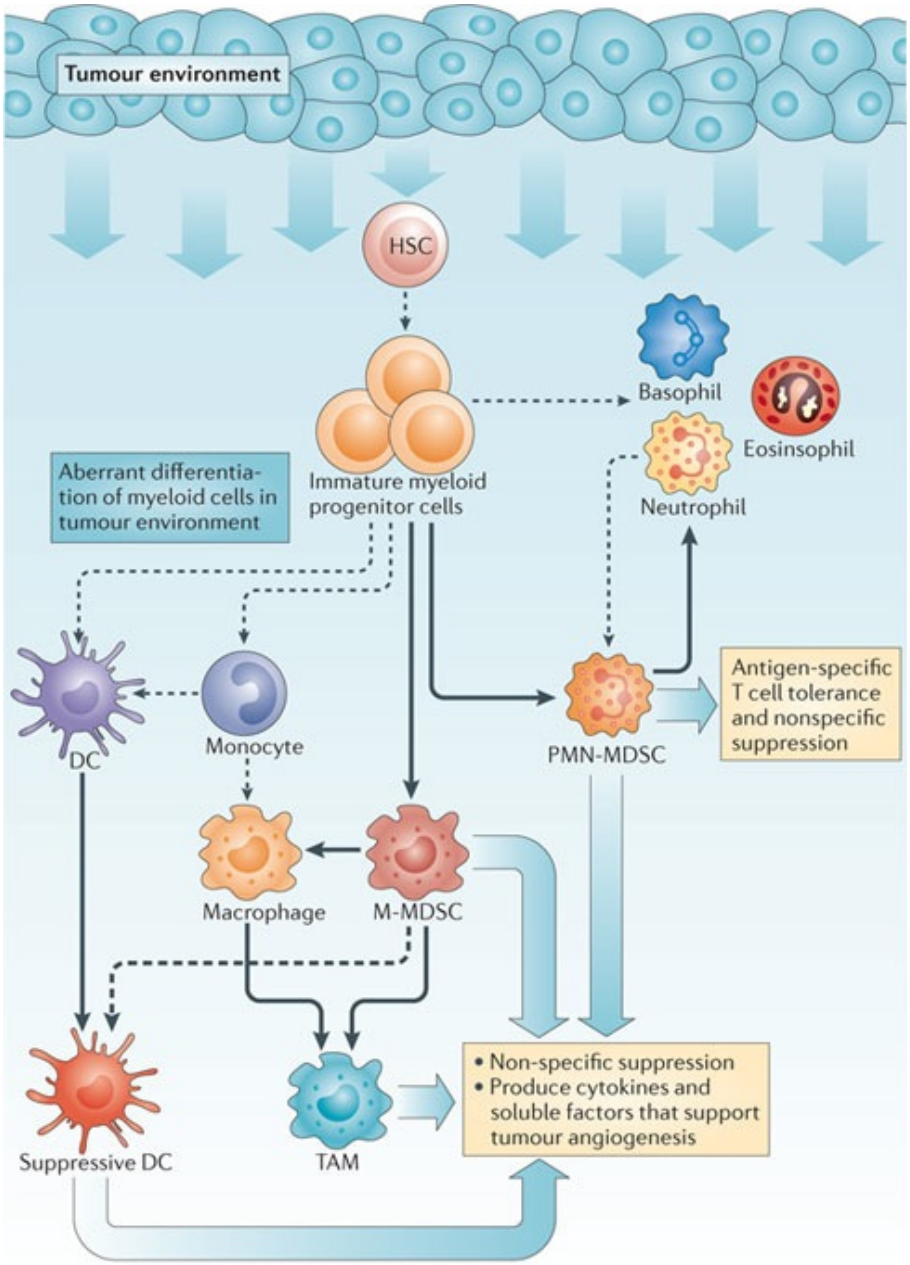


Figure 1.6: The influence of the tumour microenvironment on myeloid cell differentiation The microenvironment created by metastases promote the abnormal differentiation of myeloid cells. The bold lines indicate abnormal differentiation pathways where the tumour microenvironment promotes the development of immunosuppressive populations like MDSCs. Dotted lines indicate normal differentiation pathways (Gabrilovich DI, 2012)

populations. Cultured MDSCs can be direct progenitors of DCs, macrophages and granulocytes. After 24 hours, MDSCs cultured *in vitro* phenotypically and functionally resemble neutrophils (Youn JI, 2008), and culture of tumour-derived MDSCs without the continued influence of tumour-derived factors (Cyclooxygenase 2 (COX-2), prostaglandin, stem-cell factor (SCF), macrophage-colony stimulating factor (M-CSF), IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF)) results in the generation of macrophages and DCs (Gabrilovich DI, 2009; Fujimura T, 2010) (Figure 1.6). In the presence of tumour-derived soluble factors, MDSCs can differentiate into immunosuppressive macrophages (Narita Y, 2009). Whether immunosuppressive DCs develop through a similar mechanism has yet to be established.

1.10 The hepatic cytokine milieu

The immune cell populations resident in healthy human liver, coupled with its unique anatomical structure and state of dynamic homeostasis gives creates an unusual microenvironment laden with cytokines and growth factors. Originally described as chemical messengers attracting leukocytes towards areas of injury and inflammation, cytokines are now accepted as chemo attractants, with the potential to stimulate tumour cell migration towards sites of metastasis, creating a favourable niche in which tumour cells survive, proliferate and expand (Ali S, 2007). Healthy liver, in the absence of inflammation or infection maintains a delicate balance of both pro-inflammatory (IL-2, IL-7, IL-12, IL-15 and IFN-γ) and anti-inflammatory (IL-10, IL-13 and TGFβ) cytokines (Kelly AM, 2006). IFN-γ promotes an anti-tumour immune response by stimulating both NK and cytotoxic CD8 T+ cells whereas anti-inflammatory IL10 may facilitate tumour progression. In order for metastases to develop and grow, tumour cells must disrupt the

careful balance of pro and anti-inflammatory cytokines. Analysis of metastatic liver demonstrates increased concentrations of IFN-γ when compared to controls indicating a pro-inflammatory phenotype. A key cytokine in host defence, IFN-γ is both a regulator and effector molecule in the immune response against malignancy. In contrast, a significant correlation has been shown between high IL10 levels and decreased NK function. High levels of serum IL10 have been found in patients with colon cancer, and persistently elevated levels are associated with tumour recurrence (Galizia G, 2002). Furthermore, elevated levels of IL10 have been found in hepatic metastases which may account for suppression of the livers anti-tumour response (Kelly AM, 2006).

Several cytokines have been identified as important components of the tumour microenvironment. Critical to the development and activation of NK cells, IL15 along with IL2 signal through shared IL-2/IL-15Rβ and γ_c receptor subunits. Already in use as immunotherapy in renal cell cancer and malignant melanoma, IL2 is a central cytokine in the regulation of T cell responses (al-Ramadi BK, 2009). Previous work has shown a decrease in levels of IL15 in tumour bearing liver when compared to control. Furthermore, unchanged levels of IL2 were seen when metastatic livers were compared to control (Kelly AM, 2004). The lack of a significant increase in IL-2, produced predominantly by T cells, together with the downregulation of IL-15 levels, a key factor in development of NK and NKR^+ T cells, may contribute to the deficits in cytotoxic T cell and innate anti-tumour immune responses thus promoting tumour progression.

Despite data on cytokines and their effects on the tumour microenvironment, there remains a paucity of data regarding the cytokine profile of metastatic liver. A number of studies have focused on cytokines present at the tumour: liver interface as the driving force of metastatic success. However, little is known of the changing cytokine landscape

within the liver once metastases are established.

1.11 Current treatment strategies in the management of colorectal liver metastasis

The last two decades have seen a significant improvement in the survival of patients with metastatic CRC, partially as a result of improved screening and early detection. Improved chemotherapeutics and the introduction of monoclonal antibodies such as bevacizumab, cetuximab and pembrolizumab for the treatment of metastatic CRC has seen the median overall survival of these patients increase to 29 months (Cremolini C, 2015). Notwithstanding these advances in systemic therapy, liver resection remains the gold standard offering superior median overall survival of 56 months (de Ridder JA, 2016) and the only chance of cure. Following "curative " resection for colorectal liver metastases (CRLM) however, recurrent metastatic disease is seen in up to 62% of patients (Butte JM, 2015). Treatment strategies for CRLM patients are based on disease extent at presentation and involve multidisciplinary care to include surgery, chemotherapy and immunotherapy.

1.12 Surgical treatment of CRLM

Surgical resection of liver metastases remains the treatment of choice for localized metastatic disease, owing to the superior survival benefit conferred when hepatectomy is compared with other treatment modalities (Figure 1.7). Published series demonstrate 5-year survival rates approximating 50% following hepatectomy for CRLM (de Jong MC, 2009; Morris EJ, 2010) with acceptable surgical mortality rates of less than 2% (Wei AC, 2006). Clinical risk scores published in the 1990's demonstrated adverse post-operative outcomes when patients with more than one metastatic deposit, tumour diameters of greater than 5cm and disease involving both lobes of the undergo hepatectomy for CRLM (Fong Y, 1999; Nordlinger B, 1996).

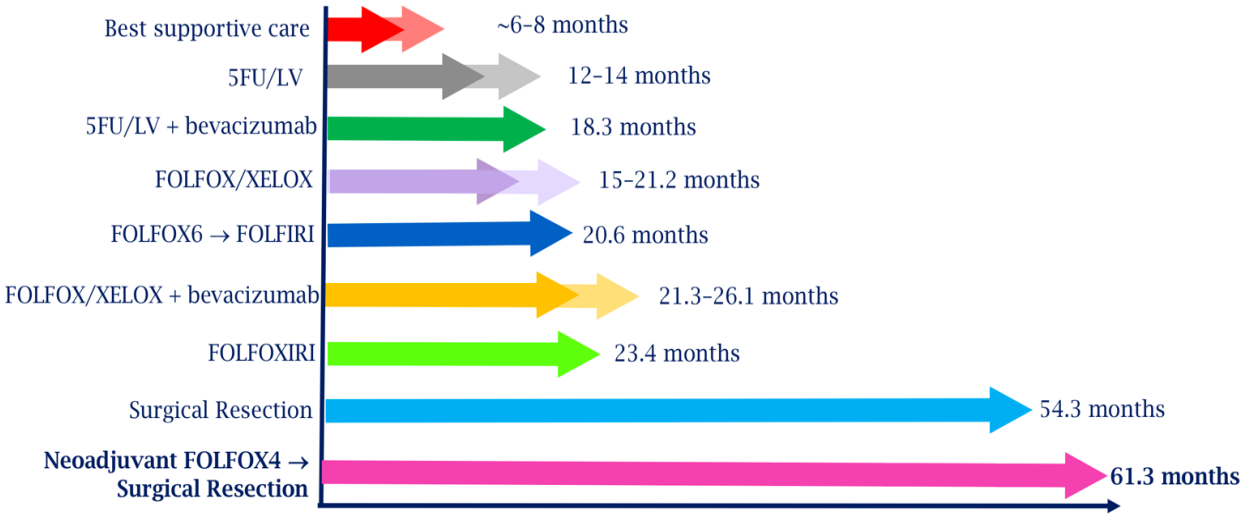


Figure 1.7 Median overall survival in patients with colorectal liver metastases treated with various modalities. For optimum outcomes, surgery with combination chemotherapy achieves a median overall survival of 61.3 months (Nordlinger B, 2013)

Additionally, advancing age (>80 years) has been identified as an adverse prognostic indicator, with 5-year survival of just 25% following hepatectomy in a retrospective series of 3957 Medicare enrolees (Robertson DJ, 2009). Despite this, optimum chemotherapeutic regimens in isolation, offer a median overall

rare. Furthermore, the clinical utility of the aforementioned clinical risk scores has been called into question with the development of more sophisticated chemotherapeutic regimens (Wimmer K, 2017; Schreckenbach T, 2015). While surgical resection remains the preferred treatment for CRLM, at initial presentation, only a fraction of patients are surgical candidates (Penna C, 2002). The main reasons cited for unresectability are technical in nature; difficult location of metastases, excessive tumour burden or predicted insufficient liver volume postoperatively. Newer techniques to manipulate liver volume such as portal vein embolization (PVE) and associating liver partition and portal vein ligation (ALPPS) coupled with improved efficacy of chemotherapy has meant that the pool of potentially resectable candidates continues to grow. Nevertheless following curative surgery where all tumours are surgically removed, metastatic disease recurs in as many as 60% of patients (Hallet J, 2016).

1.13 Chemotherapy

A number of chemotherapeutic agents are used in combination for the treatment of metastatic colorectal cancer (Table 1). When these agents are given prior to hepatic resection, it is with the aim of reducing the size of metastatic deposits, potentially enabling surgery in otherwise unfavourable cases. There is no evidence that chemotherapy administration prior to surgery prolongs overall survival in this patient cohort however (Nordlinger B, et al., 2013). Chemotherapy is given following surgery with the aim of eradicating micro-metastases and minimise disease recurrence.

Fluorouracil (5-FU) a key component in the FOLFOX combination regimen is an antimetabolite that inhibits thymidylate synthase activity in cells which is required for DNA replication. The addition of Leucovorin (LV; also Folinic Acid) increases the activity of 5-FU (Van Cutsem E, 2007). Oxaliplatin, a cytotoxic agent induces DNA cross linkages and apoptotic cell death, and when combined with 5FU and LV (FOLFOX regime) has been shown to be superior to single agents in isolation (André T, 2009). Other treatment combinations include Capecitabine/Oxaliplatin (CAPOX), 5-FU+LV, or capecitabine (oral version of 5-FU) alone for patients who cannot tolerate Oxaliplatin (Quidde J, 2012). Advantages associated with adjuvant chemotherapy have been most clearly demonstrated in patients with stage III colorectal (node-positive) cancer. In patients with metastatic colorectal cancer, while chemotherapy has been shown to increase progression free survival (Portier G, 2006), the effect on overall survival is less dramatic (Mitry E, 2008) (Nordlinger B, et al., 2013) Despite lacking demonstrable benefits, current international guidelines still recommend 6 months of adjuvant chemotherapy following metastatectomy (Van Cutsem E, 2014).

1.14 Localised therapy for CRLM

For patients with CRLM who are not amenable to potentially curative resection because of tumor size, location, or inadequate future liver remnant, localized treatments can provide an alternative to systemic chemotherapy. Similarly, where first line chemotherapy is ineffective to reduce tumour burden, just 20-35% of patients will have a demonstrable response to second line agents (Tabernero J, 2014), improved outcomes have also been shown for this patient cohort following localized treatment. Delivery of loco-regional therapy is based on tumour blood supply coming predominantly from the hepatic artery. The inverse is true in normal liver tissue, where the predominant blood

Agent	Mechanism of action
5-Fluorouracil	Inhibits thymidylate synthase activity required for DNA replication
Oxaliplatin	Inhibits DNA synthesis
Leucovorin	Induces DNA cross linkage and apoptotic cell death. Potentiates effects of 5-FU
Irinotecan	Inhibits topoisomerase I required for DNA replication
Cetuximab	Binds to and inhibits epidermal growth factor receptor (EGFR)
Bevacizumab	Inhibits vascular endothelial growth factor (VEGF-A)blocking angiogenesis
Capecitabine	Inhibits thymidylate synthase activity required for DNA replication

Figure 1.8 Chemotherapeutics used in metastatic colorectal cancer and their mechanism of action

supply is from the portal vein. In this way, localized treatments can be delivered to the tumour via the feeding hepatic artery, without compromising the blood supply to the surrounding liver. Transarterial embolization without and with chemotherapy (TACE), as is used for unresectable hepatocellular cancer, has been investigated in patients with CRLM using both conventional techniques and drug eluting beads. Limited experience suggests that hepatic arterial embolization and TACE can achieve disease stabilization in 40-60% of patients, however, whether this translated into an improvement in overall survival is less clear. Most studies investigating the efficacy of chemoembolization in this setting lack a control group, (Martin RC, 2011) (Bhutiani N, 2016). An early trial randomly assigned 61 patients with CRLM to hepatic artery embolization, hepatic artery infusion chemotherapy, or a control group in which no active intervention was undertaken. When compared to the control group, no significant benefit was seen with hepatic artery embolization (7 versus 7.9 months' median overall survival), however, aside from 5FU, this trial predated the advent of targeted chemotherapeutic agents.

Radiofrequency ablation (RFA) is a localised thermal treatment, administered directly to the metastasis inducing tumour destruction by heating the tissue to temperatures over 60℃. An alternating current of radiofrequency waves are passed from an uninsulated electrode into surrounding tissues. These radiofrequency waves instigate frictional heating, ionic agitation and subsequent tissue heating, which in turn causes coagulative necrosis. RFA is recommended for liver metastases with a maximum diameter of 3cm, in patients with no more than 3 tumour deposits, however the maximum number of metastatic deposits is not an absolute if all deposits can potentially be ablated successfully. At present, there are no randomized controlled trials analyzing disease free

interval or overall survival comparing RFA to surgery. However, retrospective studies of several cohorts have described diverse outcomes (Reuter NP, 2009; Han Y, 2016). Reuter et al described a series of 192 patients with CRLM who underwent either RFA or hepatic resection. While the numbers of hepatic lesions were similar between both groups, the median time to tumour recurrence was shorter in the ablation group (12.2 months) versus 31.1 months in the surgical group. Similarly, tumour recurrence at the site of ablation was higher post RFA (17% versus 2% recurrence of tumour at previous resection site). Further tumour development within the ablated liver was also more common when compared to resection (33% of patients post RFA, versus 14% post resection) (Reuter NP, 2009).

Yttrium 90 radioembolization or Selective Internal Radiation therapy (SIRT) delivers a targeted radiation dose to the liver via the hepatic artery. These microspheres pass through the arteries and become preferentially lodged in the smallest blood vessels of the tumour where they release radiation to surrounding tissue. Their small size prevents these microspheres from passing through the tumour vasculature and into the venous circulation. Early randomized controlled trials demonstrated efficacy of SIR-Spheres Y-90 resin microspheres when added to first-line chemotherapy, significantly increasing overall survival in patients with CRLM (Gray B, 2001). More recently, though combined results of the SIRFLOX/FOXFIRE prospective randomized studies have demonstrated a significant benefit in liver specific progression free survival, no increase in overall survival was demonstrated with SIRT and FOLFOX in combination (Wasan HS, 2017).

1.15 Molecular targeted therapy of CRLM

Recent developments in medical oncology have concentrated on patient-specific tailored therapy, whereby definitive molecules, present in the developing tumour are

manipulated to limit tumour growth. Vascular endothelial growth factor (VEGF) is a key growth factor involved in tumour initiated angiogenesis. It stimulates intratumoural micro vessel proliferation (neoangiogenesis), facilitating malignant dissemination by increasing blood vessel permeability and promoting vascular invasion. VEGF expression has been shown to be upregulated in colonic tissue of patients with CRC and therefore has been shown to be of prognostic value (Goi T, 2015). Bevacizumab, a monoclonal antibody directed against VEGF-A when combined with standard chemotherapy, potentiates its effects in the treatment of metastatic CRC patients (Loupakis F, 2014). Overall, response rates and median overall survival improved by 2-5 months when Bevacizumab was added to standard treatments such as IFL (Irinotecan/ fluorouracil/ leucovorin), FOLFOX, or FU/LV (Giantonio BJ, 2007; Guan ZZ, 2011). However, not all patients respond to Bevacizumab, highlighting the need for predictive biomarkers in this cohort.

Epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, activates the Ras-Raf-MEK-ERK and PI3K pathways when bound to its ligand to promote tumour growth and survival. Mutations in downstream effectors and regulators (KRAS/NRAS mutations) can confer growth and survival advantages (Grossmann AH, 2011). Panitumumab and Cetuximab, monoclonal antibodies that selectively bind the EGF receptor play a role in controlling cellular growth and differentiation (El Zouhairi M, 2011). In patients without a KRAS gene mutation, the addition of Cetuximab has dramatically increased response rates to standard chemotherapy and has improved overall survival rates (Van Cutsem E, 2011). However, KRAS mutations predict adverse outcomes with cetuximab treatment, along with BRAF, NRAS and PIK3CA exon 20 mutations which are equally associated with poor response rates (De Roock W, 2010).

In patients whose disease has progressed on standard chemotherapy, regorafenib a multikinase inhibitor with several targets including anti-angiogenesis has been shown to significantly increase overall survival (Grothey A, 2013). Interestingly, these results were replicated in a phase 3 trial from Asia demonstrating median overall survival of 8.8 months in the regorafenib group versus 6.3 months following placebo (Li J, 2015).

1.16 Immunotherapeutic treatment of CRLM

Immunotherapy has shown promising results in the treatment of melanoma, bladder cancer and non-small cell lung cancer. Consequently, trials are now underway in patients with colorectal malignancy. In total, 5 immune checkpoint molecules have been identified as potential targets for immune therapy. These include programmed cell death (PD)-1, PD ligand 1 (PD-L1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), lymphocyte activation gene (LAG-3) and indoleamine 2,3-dioxygenase (IDO). Thus far however, only CTLA-4 and PD-1 inhibitors have been used in patients with colon cancer with limited success perhaps due to the complexity of the local immune repertoire in the intestine. Programmed cell death-1 (PD-1) is a cell surface receptor found on activated and exhausted T cells, macrophages and B cells (Wu X, 2014).

Once PDL-1 binds to PD-1 it induces a negative feedback loop for adaptive immunity, suppressing Th1 activity (Chen L, 2015). In the cancer setting, expression of PD-1 is upregulated on tumour infiltrating lymphocytes suppressing their anti-tumour cytotoxic T cell activity. PD1 receptor blockade using drugs such as pembrolizumab and nivolumab has been successful in achieving a clinical response in a number of malignancies including melanoma, Hodgkin's lymphoma as well as renal cell and bladder cancer (Quéro L, 2019; Queirolo P, 2019). However, the response in colon cancer has been less dramatic. Nivolumab was administered to 296 patients with varying

malignancies in the phase 1 study. While microsatellite status was unknown prior to the trial commencement, none of the colorectal cancer patients demonstrated a response to nivolumab (Topalian SL, 2012). In the phase II study, patients were administered either nivolumab monotherapy or nivolumab in combination with ipilimumab, microsatellite status was known prior to trial commencement. While no patient demonstrated complete response to therapy, a partial response was seen in 25.5% of MSI patients with no response in the microsatellite stable group (MJ Overman, 2018).

There is a demonstrable difference in the immunogenic response incited by MSI tumours when compared to MSS tumours, this is currently the subject of multiple active clinical trials involving anti-PD1 and anti PDL-1 therapy (Passardi A, 2017). While the phase 2 trials demonstrated some evidence of tumour regression in MSI tumours, this was in a subset of patients. Colorectal tumours that develop liver metastases are frequently either microsatellite stable, or express low levels of microsatellite instability, highlighting the lack of immunologic biomarkers identified in the treatment of metastatic colorectal cancer.

The immune landscape of colorectal tumours and their corresponding metastases varies greatly, the reasons for which are largely unknown. Differences in metabolism, genetic makeup or epigenetics between cancer cells in the primary tumour versus metastases are likely to contribute to their heterogeneity. Furthermore, how cancer cells react to the host tissue and its local immune microenvironment likely shapes their response to targeted immunotherapy. A fundamental understanding of the immune microenvironment of healthy human liver and how it is manipulated in the presence of metastases is critical to tackle metastases in individual patients, and strive towards personalised immune interventions.

1.16 Rationale for thesis

Approximately 50% of patients with CRC develop liver metastases during the course of their disease with metastases the chief determinant of life expectancy. While surgical resection of metastatic deposits remains the optimum treatment, less than 30% of those diagnosed with CRLM are suitable for surgical resection (Khatri VP, 2005). Furthermore, of those that undergo curative hepatectomy, some 60-70% of patients will suffer disease recurrence (Ali MA, 2015; Heise D, 2017; Neal CP, 2017). Healthy liver maintains homeostasis via a complex landscape of immune cells, cytokines and chemokines. The development of metastases manipulates this landscape, creating a favourable immune microenvironment for tumour growth. Analysis of this microenvironment will identify immunologic targets, allowing a targeted approach to the treatment of CRLM. In this thesis, we will describe the Irish population of patients who have undergone liver resection for CRLM in the last decade, in an attempt to stratify patient and tumour factors that may have an effect on postoperative outcome; we will then characterise the immune cells, cytokines and chemokines present in metastatic liver. in this way we will identify factors that might influence local antitumour responses during the development of CRLM and help predict tumour recurrence.

1.17 Hypothesis

We propose the immune homeostasis of healthy liver is manipulated in the formation of hepatic metastases. For sustained tumour development, we believe the hepatic microenvironment is altered permitting hepatic immune cells, cytokines and chemokines to create a pro-metastatic niche. Following surgery, without re-establishment of the livers healthy immune microenvironment, the liver is vulnerable to recurrent metastasis.

1.18 Overall objectives

a) To characterise the Irish cohort of patients undergoing hepatectomy for CRLM and identify patient and tumour variables which may be associated with shorter overall survival.

b)To characterise the immune cell composition of metastatic liver and ascertain possible immunotherapeutic targets.

c)To establish what cytokines are present in CRLM that potentiate the pro-tumorigenic niche present in metastatic liver.

d)To ascertain if the immune profile of metastatic liver differs between patients that develop recurrent metastases and those that remain disease free.

Chapter 2

Preoperative neutrophil to lymphocyte ratio predicts postoperative survival in patients with colorectal liver metastases

2.1 Introduction

The development of metastatic disease remains the chief determinant of mortality in patients with CRC, with hepatic metastases the most common to develop (Nathan H, 2010). Surgical resection remains at the cornerstone of optimum treatment regimens with the addition of perioperative chemotherapy affording a 5-year survival approaching 60% (Nordlinger B, 2013). Despite this, numerous published series have outlined high rates of disease recurrence following hepatectomy with further metastases seen in up to three quarters of patients (Heise D, 2017; Neal CP, 2017). The last decade has seen a marked increase in the number of patients being referred to the National Liver Unit at St. Vincent's Hospital for resection of CRLM. Nonetheless, Irish data is lacking regarding the number of patients undergoing index resection for CRLM, their outcomes or indeed the number that return for repeat hepatectomy. International reports suggest just 23% of patients remain disease free at 10 years post hepatectomy (Pulitanò C, 2010), underscoring the need to accurately stratify these patients at diagnosis.

Clinical risk scores developed in the 1990's highlighted a number of prognostic variables for index hepatectomy including patient age, number of metastatic deposits and resection margin (Nordlinger B, 1996; Fong Y, 1999). However, since the publication of these clinical risk scores, the surgical approach to CRLM and therapeutic treatment algorithm has changed dramatically. Furthermore, in light of contemporary treatment paradigms, the clinical utility of these scores has been questioned (Schreckenbach T, 2015).

Preexisting inflammation arising from infection, alcohol and excess fat deposition form a well-established pathway to hepatocellular carcinoma. In contrast, colorectal liver metastases (CRLM) form in what is clinically a healthy uninflamed hepatic microenvironment. The progression of metastases is known to induce a local

inflammatory response, disrupting hepatic homeostasis in order to potentiate further malignant growth (Albillos et al, 2014). The systemic inflammatory response has been postulated to play a key role in this, facilitating tumour progression and the development of metastasis (Proctor MJ, 2010). Elevated neutrophil to lymphocyte (NLR) ratios correlate with poor disease specific and overall survivals in a number of solid organ malignancies (Sierzega M, 2017; Templeton AJ, 2014; Tsai PL, 2016). Proposed as a systemic barometer of this malignancy induced inflammation, high NLR has been associated with a specific pro-inflammatory cytokine signature in plasma, and poorer overall survival in the setting of colorectal liver metastases (Tang H, 2016).

The aim of this chapter was to:

1. Describe the Irish cohort of patients undergoing hepatectomy for colorectal liver metastases
2. Establish the overall survival rates following hepatectomy for CRLM
3. Analyze preoperative systemic inflammatory indices to ascertain prognostic significance
    i) for index hepatectomy
    ii) following administration of neoadjuvant chemotherapy
    iii) for repeat hepatectomy

2.2 Materials and methods

2.2.1 Study design

In order to characterize the Irish cohort of patients undergoing hepatic resection for CRLM, a retrospective study of all patients referred for hepatic resection to St. Vincent's University Hospital was performed. A contemporaneous database of all liver resections is maintained by the hospitals Pathology Department including patient identifiers, date of surgery and tumour type. This database was interrogated for all patients, and those with colorectal liver metastases who underwent hepatic resection between 1st January 2005 and 31st December 2014 were selected out for further data mining. As no electronic patient record exists in St Vincent's Hospital, demographic and clinicopathological details were sought from patient charts and clinical records. Blood neutrophil and lymphocyte counts obtained from patient's routine full blood counts at the preoperative assessment clinic were sought from the hospitals laboratory results system. No patient had clinical evidence of active infection or inflammation at the time of blood sampling. Follow up data and specific details of postoperative care were sought from the patients referring institute and, in some cases from the patient's primary care physician.

2.2.2 Setting

The Liver Unit at St. Vincent's University Hospital is the national referral unit for liver surgery in Ireland. Cases of liver and pancreas malignancy are referred from regional centres around Ireland and discussed at a weekly multidisciplinary conference attended by members of medical oncology, radiology (interventional and diagnostic), hepatopancreaticobiliary surgery and histopathology. Similarly, patients diagnosed with colorectal liver metastases, often having diagnostic imaging performed in their own local hospital then have their images referred for multidisciplinary discussion. Full review of

diagnostic imaging and histology (if tissue has been obtained at biopsy) is performed by five consultant surgeons. Of those referred, approximately 55- 60 patients with CRLM are deemed suitable for resection and undergo metastatectomy per annum. Despite the availability of statistics on CRC diagnoses nationally, to date there remains no national data on the proportion of patients diagnosed with colorectal liver metastases each year in Ireland. Furthermore, operative outcomes of patients referred to the national liver unit with CRLM are as yet unknown.

2.2.3 Recruitment

From January 2005 to January 2015, a review of all hepatectomies performed at the national liver unit was conducted. Patients with a histological diagnosis consistent with metastases from a primary CRC were included in our database for further analysis. Details of all relevant patients were sought and included demographics (age, sex, date of surgery), preoperative haematological parameters (neutrophil and lymphocyte count), histological tumour characteristics (number and size of metastatic deposits, differentiation, treatment effect) give specifics, histological characteristics of hepatic parenchyma (steatosis, fibrosis, cirrhosis) and, where available treatment modalities. All patients underwent preoperative staging comprising computed tomography of the chest, abdomen, and pelvis and primovist enhanced magnetic resonance imaging of the liver. Following review of their imaging, patients were discussed at a weekly multidisciplinary team conference and a therapeutic strategy was decided upon.

2.2.4 Surgical technique

For all cases, the liver parenchyma was dissected using an ultrasonic dissector, with haemostasis achieved using diathermy, and suturing. If necessary, intermittent clamping of the portal triad (the Pringle manoeuvre) was performed during parenchymal dissection,

with periods of occlusion of up to 10 minutes alternating with 5-minute release intervals. After hepatic resection, patients were transferred to the intensive care unit or the high-dependency unit, where an established clinical care protocol was followed.

2.2.5 Inclusion criteria

All consecutive patients undergoing hepatic metastatectomy for CRLM between 1 January 2005 and 31 December 2014 were included in this study in this analysis**.**

2.2.6 Exclusion criteria

Exclusion criteria included patients undergoing hepatic resection for indications other than CRLM e.g. hepatocellular carcinoma, cholangiocarcinoma. Furthermore, those who had their primary tumour still *in situ* at hepatectomy, planned 2-stage hepatectomies, planned synchronous resection of colonic primary at the time of hepatectomy, resection of extrahepatic disease at the time of hepatectomy and those who had a complete response to neoadjuvant chemotherapy with no residual malignancy on final histology were all excluded from our analysis.

2.2.7 Ethics

This study was approved by the research and ethics committee of St. Vincent's Hospital. All protocols were in accordance with the guidelines of the 1975 declaration of Helsinki. Informed written consent was obtained from all study participants prior to undergoing surgical resection.

2.2.8 Data retrieval and management

Medical records provided the chief source of patient data. For those local to the hospital,

clinical records were in the main complete with both oncological and surgical data and an accurate record of the patient's current clinical status. However, as St Vincent's Hospital is the national center for liver surgery, more than 80% of patients are referred from other institutions. Consistent data pertaining to these patient's oncology treatments and primary cancer surgery, often performed elsewhere is lacking. Furthermore, once these patients are discharged home following surgery, follow up is usually carried out locally thus, long term survival data is unknown. In all cases where the medical record in St. Vincent's was incomplete, information was sought from either the referring team of record, the National Integrated Medical Imaging System (NIMIS), or contact with local community oncology or palliative care teams. Confirmation of survival for all patients was achieved by telephone contact with the patient's primary care physician of record. All data obtained was entered prospectively on a customized excel database on a password protected computer in the National Liver Unit. Similarly, access to the database was password protected. Each patient was assigned a study number, with the study code known only to the principal researcher.

2.2.9 Sample collection

Each patient's surgical specimen and corresponding preoperative blood sample was reviewed for analysis. All patients prior to surgery were required to attend an anaesthetic review at the preoperative assessment clinic. This review took place on average 7-10 days prior to surgery and 6 weeks post the completion of chemotherapy. Full blood counts obtained for each patient were processed in the hospital laboratory and quantities of lymphocytes and neutrophils were noted, in order to calculate each patients NLR. Details of surgical specimens retrieved at hepatectomy were also reviewed for analysis. Sample size, tumour size and number of metastases were documented along with grade of differentiation (poor, moderate, well differentiated). Furthermore, specific details on the

uninvolved liver were sought; presence of cirrhosis, fibrosis or steatosis.

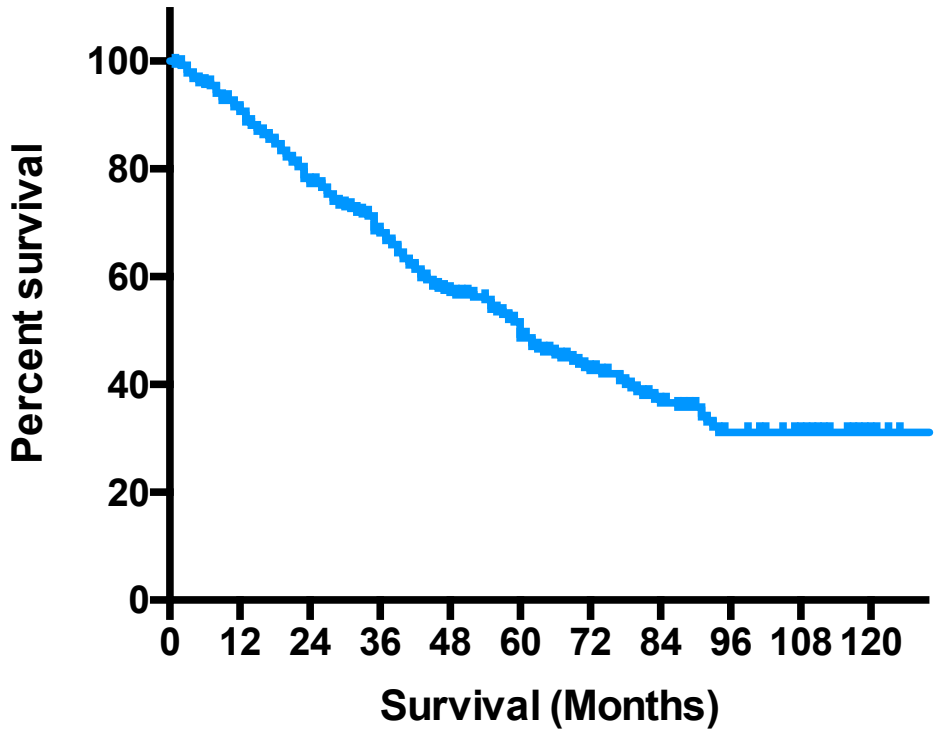
2.2.10 Statistical analysis

Overall survivals were assessed by Kaplan Meier methods. Univariate and multivariate logistic regression analyses were used to examine the effect of variables on long term mortality postoperatively. Univariate associations with long-term survival were determined using Cox regression analysis, Kaplan-Meier analysis, and the log-rank test. Multivariate analyses were performed by Cox proportional hazards regression analysis (using a stepwise backward procedure), incorporating all variables with $P < .15$ on univariate analysis. Statistical significance was defined as $P < .05$. These analyses were conducted using a software program (SPSS version 16.0; SPSS, Inc, Chicago, Illinois).

2.3 Results

Five hundred and four consecutive patients underwent hepatic metastatectomy in the study time period. Applying exclusion criteria, 379 patients were suitable for inclusion in this analysis, 322 who underwent an index hepatectomy and 57 second hepatectomies. All surgeries were performed with curative intent. The median follow-up following index hepatectomy was 41 months with 1-, 3- and 5-year survival rates of 90.7%, 68.1% and 48.6% respectively (median 59 months). Of those undergoing index hepatectomy, 205 (63.7%) were male, with 252 (78.3%) of the cohort younger than 70 years. The median number of tumours in the index group was 1 (range 1-14), with 118 patients (36.6%) undergoing major hepatectomy, defined as resection of three or more Couinaud segments (Table 1).

Univariate analysis of index hepatectomies revealed tumour diameter greater than 5cm, poor tumour grade, positive resection margins and increased number of metastatic deposits were all significantly associated with shorter postoperative survival (Table 1). The use of preoperative chemotherapy however, showed no correlation with overall survival (p=0.093). In addition, elevated neutrophil lymphocyte ratio and independent neutrophil counts had a negative correlation with overall survival on univariate analysis (p=0.027, p=0.018 respectively).



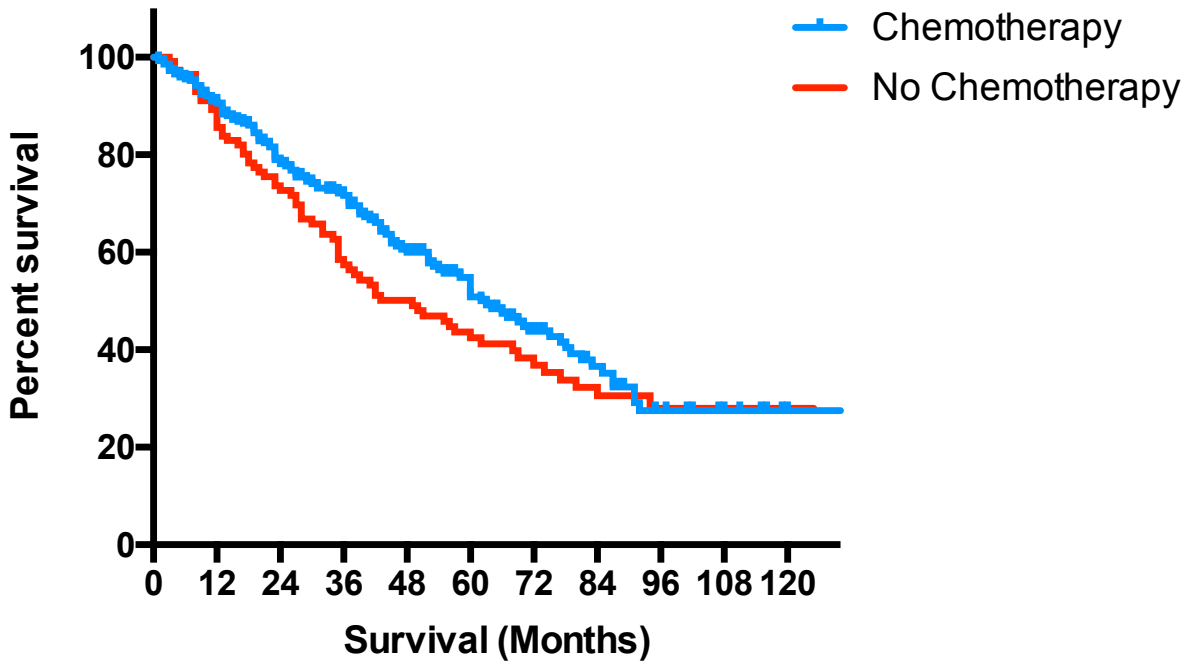
	0 months	6 months	12 months	36 months	60 months
Number of Patients at Risk	322	319	299	194	105

Figure 2.1 Survival of all index hepatectomies between 1st January 2005 and 31st December 2014

Table 2.1 Univariate analysis (Kaplan-Meier) of patient variables and overall survival for all colorectal liver metastatectomies performed from 1st January 2005 to 31st December 2014. Exclusions applied; 30-day mortality, absent tumour on histology, repeat hepatic resections, combined colon hepatic resections, liver first resections.

Characteristic	n (%)	Overall Survival (p-value*)
Neoadjuvant Chemotherapy (no/yes)	85 (29.6)/202 (70.4)	0.093
Gender (m/f)	205 (63.7)/117 (36.3)	0.885
Age (<70/70+)	252 (78.3)/70 (17.7)	0.052
Tumour Number (1/ 2-5 / >5)	179(56.1)/ 118(37.0)/ 22(6.9)	**<0.001**
Segmentectomy (Minor/Major)	204 (63.6)/117 (36.4)	**0.040**
Tumour Grade (Poor/Mod-Well)	40 (12.4)/ 282 (87.6)	**0.026**
Tumour Max Diameter (<30/ 30-50 / >50mm)	142 (44.1)/108 (33.5)/72 (22.4)	**<0.001**
Margins (R0/R1-R2)	274 (85.1)/48 (14.9)	**0.002**
LVI (no/yes)	219 (73.5) / 79 (26.5)	0.981
Steatosis (no/yes)	264 (82.0) / 58 (18.0)	0.166
Neutrophil Threshold (<8/8+)	266 (82.6)/ 56 (17.4)	**0.018**
Lymphocyte Threshold (<1.5/1.5+)	174 (54.0) /148 (46.0)	0.256
NLR Threshold (<5/5+)	221 (68.6) /101 (31.4)	**0.027**

When stratified according to the receipt of neoadjuvant chemotherapy, similar overall survival was noted between those that received chemotherapy in the preoperative setting, and those that were chemotherapy naïve at surgery (p=0.18, Figure 2.2). Survival at 1-, 3- and 5-years in those post neoadjuvant chemotherapy was 90.6%, 71.6% and 50.8% as compared to 85.7%, 72.7% and 42.5% in the chemotherapy naïve group. Neither neutrophils, lymphocytes nor neutrophil lymphocyte ratio were predictive of overall survival in the neoadjuvant chemotherapy group (p=0.37, p=0.47, p=0.93 respectively, Table 2.2). An inverse trend however was noted in the chemotherapy naïve group, with all three variables predictive of outcome (Table 2.2). Multivariate analysis confirmed an independent association between elevated preoperative neutrophil count and shortened overall survival after index metastatectomy, however, no such association was found for NLR. Similarly, patient age, tumour size, tumour number and margin status all demonstrated prognostic significance (Table 2.3). However, neither segmentectomy nor tumour grade showed prognostic significance on multivariate analysis for overall survival.



Number of Patients at Risk	0 months	6 months	12 months	36 months	60 months
Neoadjuvant chemotherapy	202	195	183	145	103
Chemotherapy naïve	85	83	73	49	36

Figure 2.2 Kaplan Meier Curve representing the overall survival probability for patients following index hepatectomies stratified according to receipt of preoperative chemotherapy between 1st Jan 2005 and 31st Dec 2014. Statistical analysis performed using log rank test, p=0.18

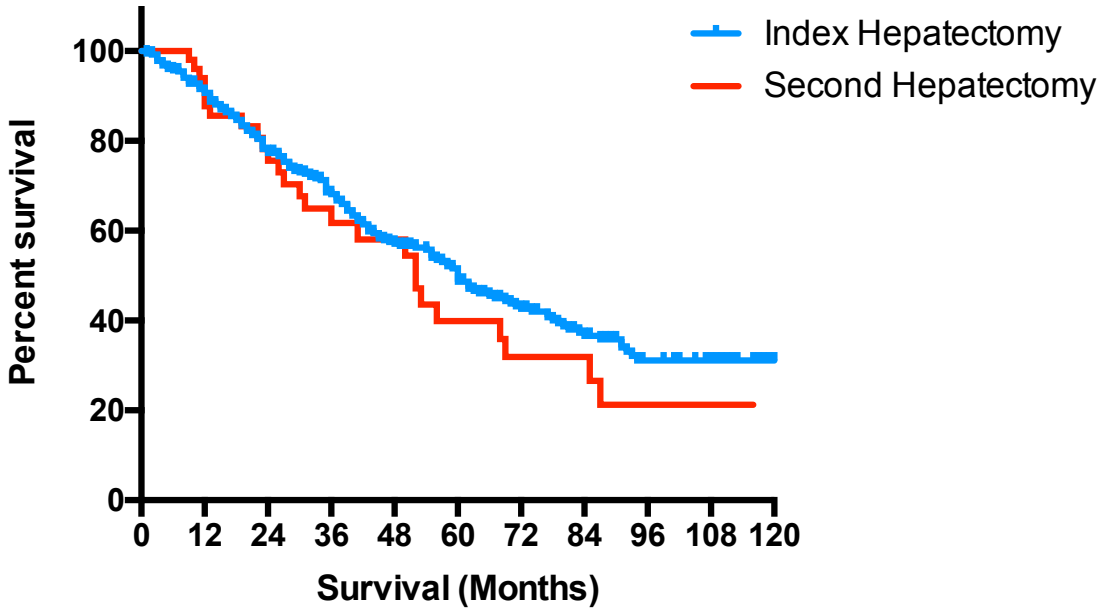
Table 2.2 Univariate Analysis (Kaplan Meier) of patient variables and overall survival for all colorectal liver metastatectomies performed (1st January 2005 - 31st December 2014). Patients were stratified according to the receipt of neoadjuvant chemotherapy. Exclusions applied were 30-day mortality, absent tumour on histology, combined colon hepatic resections, liver first resections.

Characteristic	Neoadjuvant Chemotherapy (n=202) n (%)	p-value	Chemotherapy Naïve (n=85) n (%)	p-value
Gender				
Male	123 (60.9	0.713	58 (68.2)	0.756
Female	79 (39.1)		27 (31.8)	
Age				
<70	161 (79.7	0.644	63 (74.1)	0.072
>70	41 (20.3)		22 (25.9)	
Tumour Number				
1	106 (52.5)	**0.035**	55 (65.5)	**0.002**
2-5	81 (40.1)		26 (31.0)	
>5	15 (7.4)		3 (3.5)	
Segmentectomy				
Minor	141 (69.8)	0.410	49 (58.3)	0.541
Major	61 (30.2)		35 (41.7)	
Tumour Grade				
Poorly Differentiated	23 (11.4)	0.447	12 (14.1)	**<0.001**
Moderate-Well Differentiated	179 (88.6)		73 (85.9)	
Tumour Max Diameter				
<3cm	97 (48.0)	**0.005**	32 (37.6)	**0.004**
3-5cm	69 (34.2)		29 (34.1)	
>5 cm	36 (17.8)		24 (28.2)	
Margins				
R0	169 (83.7)	**0.012**	75 (88.2)	0.300
R1-R2	33 (16.3)		10 (11.8)	
Lymphovascular Invasion				
Yes	137(67.8)	0.991	63 (74.1)	0.638
No	57(28.2)		17(20)	
Unknown	8(4)		5(5.9)	
Steatosis				
Yes	166 (82.2)	0.132	67 (78.8)	0.892
No	36 (17.8)		18 (21.2)	
Neutrophil Threshold				
<8	165 (81.7)	0.377	72 (84.7)	**0.012**
>8	37 (18.3)		13 (15.3)	
Lymphocyte Threshold				
<1.5	114 (56.4)	0.474	44 (51.8)	**0.002**
>1.5	88 (43.6)		41 (48.2)	
NLR Threshold				
<5	139 (68.8)	0.935	56 (65.9)	**0.017**
>5	63 (31.2)		29 (34.1)	

Table 2.3 Multivariate analysis of all index hepatectomy patient variables and overall survival using Cox Regression Survival Analysis (Exclusions: 30-day mortality, absent tumour on histology, repeat hepatic resections, combined colon hepatic resections, liver first resections)

Characteristic	Hazard Ratio (95% CIs)	Overall Survival (p-value)
Age	1.020 (1.002 to 1.038)	**0.033**
Segmentectomy (Minor/Major)	1.018 (0.717 to 1.446)	0.919
Tumour Grade (Poor/Mod-Well)	1.156 (0.711 to 1.881)	0.559
Tumour Max Diameter (<3/ 3-5 / >5 cm)	2.675 (1.793 to 3.990)	**<0.001**
Tumour Number (1/ 2-5 / >5)	2.461 (1.332 to 4.546)	**0.004**
Margins (R0/R1-R2)	1.765 (1.103 to 2.825)	**0.018**
Neutrophil	0.921 (0.875 to 0.968)	**0.001**

Following index hepatectomy, 57 patients (15%) developed recurrent intrahepatic metastases deemed suitable for repeat resection in the study time period. Median follow-up following repeat hepatectomy was 36.4 months with 1-, 3- and 5-year survival of 87.9%, 61.7% and 39.9% respectively (median 52 months), with no significant increased mortality when compared to index hepatectomy (p=0.35, Figure 2.3). On univariate analysis, neither serum neutrophil nor lymphocyte count correlated to overall survival in those undergoing second hepatectomy (p=0.42, p=0.97 respectively, Table 2.4). Similarly, neutrophil lymphocyte ratio demonstrated no significant effect on outcome following repeat resection (p=0.81) However, female gender, tumour diameter >5cm and major segmentectomy were all predictive of poorer overall survival on univariate analysis (Table 2.4). As with the index hepatectomy cohort, no significant difference in survival was noted between chemotherapy naïve patients and those that received neoadjuvant therapy. On multivariate analysis, tumour diameter >50mm was the only variable independently associated with worse overall survival in those undergoing second hepatectomy (Table 2.5).



Number of Patients at Risk	0 months	6 months	12 months	36 months	60 months
Index hepatectomy	322	308	292	220	157
Repeat hepatectomy	57	57	50	35	23

Figure 2.3 Kaplan Meier Curve showing overall survival stratified according to index and second hepatectomies performed between 1st Jan 2005 and 31st Dec 2014, Statistical analysis performed using log rank test, p=0.45

Table 2.4 Univariate analysis (Kaplan-Meier) of patient variables and overall survival for all colorectal liver metastatectomies performed from 1st January 2005 to 31st December 2014. Patients were stratified according to index hepatectomy versus second hepatectomy for recurrent disease. Exclusions applied were 30-day mortality, absent tumour on histology, combined colon hepatic resections, liver first resections.

Characteristic	Index Hepatectomy n=322 n(%)	p-value	Second Hepatectomy n=57 n(%)	p-value
Gender				
Male	205 (63.7)	0.885	38 (66.7)	**0.046**
Female	117 (36.3)		19 (33.3)	
Age				
<70	252 (78.3)	0.052	48 (84.2)	0.584
>70	70 (17.7)		9 (17.8)	
Neoadjuvant Chemotherapy				
Yes	202(62.7)	0.093	41(71.9)	0.432
No	85(26.4)		14(24.6)	
Unknown	35(10.9)		2(3.5)	
Tumour Number				
1	180(56.1)	**<0.001**	32 (56.1)	0.388
2-5	119(37.0)		24 (42.1)	
>5	23(6.9)		1 (1.8)	
Segmentectomy				
Minor	204 (63.4)	**0.040**	43 (75.4)	**0.035**
Major	118 (36.6)		14 (24.6)	
Tumour Grade				
Poorly Differentiated	40 (12.4)	**0.026**	13 (22.8)	0.832
Moderate-Well Differentiated	282 (87.6)		44 (77.2)	
Tumour Max Diameter				
<3cm	142 (44.1)	**<0.001**	22 (38.6)	**0.007**
3-5cm	108 (33.5)		26 (45.6)	
>5 cm	72(22.4)		9 (15.8)	
Margins				
R0	274 (85.1)	**0.002**	41 (71.9)	0.375
R1-R2	48 (14.9)		16 (28.1)	
Lymphovascular Invasion				
Yes	79(24.5)	0.981	20(35.1)	0.419
No	219(68)/		35 (61.4)	
Unknown	24(7.5)		2(3.5)	
Steatosis				
Yes	58 (18.0)	0.166	35 (61.4)	0.749
No	264 (82.0) /		22 (38.6)/	
Neutrophil Threshold				
<8	266 (82.6)	**0.018**	49 (86.0)	0.428
>8	56 (17.4)		8 (14.0)	
Lymphocyte Threshold				
<1.5	174 (54.0)	0.256	31 (53.8)	0.975
>1.5	148 (46.0)		26 (46.2)	
NLR Threshold				
<5	221 (68.6)	**0.027**	44 (77.2)	0.815
>5	101 (31.4)		13 (22.8)	

Table 2.5. Multivariate analysis of patient variables and overall survival following second hepatectomy using Cox Regression Survival Analysis (Exclusions: 30-day mortality, absent tumour on histology, index hepatic resections, combined colon hepatic resections, liver first resections)

Characteristic	**Hazard Ratio (95% CIs)**	**Overall Survival (p-value)**
Age	1.021 (0.972 to 1.072)	0.410
Gender	2.204 (0.825 to 5.887)	0.115
Segmentectomy (Minor/Major)	1.539 (0.589 to 4.017)	0.379
Tumour Max Diameter (<3/ 3-5 / >5 cm)	3.292 (1.006 to 10.771)	**0.049**

2.4 Discussion

Surgical resection remains the only curative option for patients who develop colorectal liver metastases. Nonetheless, this study is the first to describe hepatic resection for colorectal liver metastases in an Irish setting. From our analysis, overall patient survival at 1-, 3- and 5-years following hepatectomy in Ireland is comparable to that quoted in international literature (Gur I, 2013). This present work however raises a number of key points in the management strategy for colorectal liver metastases at a national level. Our analysis of the Irish cohort demonstrated a number of factors with prognostic significance for overall survival. On univariate analysis, larger metastases, increased number of metastatic deposits and R2 margins were all significantly associated with poorer long-term survival. Interestingly however, the use of neoadjuvant chemotherapy was not associated with longer survival post hepatectomy.

Increasingly being offered to patients presenting with metastatic disease, neoadjuvant chemotherapy has been shown to reduce tumour bulk in the region of 50-80% (García-Alfonso P, 2015) with the additional benefit of treating occult micrometastases. In primarily unresectable patients, chemotherapy has been shown to convert 10-30% into surgical candidates (Adam R, 2004). Where tumours are resectable at presentation however, the use of chemotherapy in the neoadjuvant setting remains controversial owing to increased incidence of hepatic parenchymal injury (Viganò L, 2013). Furthermore, tumour response to preoperative chemotherapy has not been shown to correlate with overall patient survival post resection (Gallagher DJ, 2009) leading to a number of institutions offering upfront hepatic resection if feasible at diagnosis (Nanji S, 2013). From analysis of our cohort, the majority of patients (62.7%) underwent neoadjuvant chemotherapy, with larger sized tumours, increasing number of metastases and R2

resection margins all associated with shorter survival. Interestingly, inflammatory indices were not predictive of outcome in the neoadjuvant chemotherapy group. However, lymphocytes, neutrophils and neutrophil: lymphocyte ratio were all predictive of survival in the chemotherapy naïve group on univariate analysis.

Systemic inflammation has been linked to poorer outcomes across multiple types of solid organ malignancies, notwithstanding colorectal liver metastases (Malietzis G, 2014). Postulated to reflect a global inflammatory response to malignant invasion this reaction serves to facilitate cancer cell proliferation and metastasis by promoting angiogenesis and inhibiting apoptosis (Jaiswal M, 2000; McMillan DC, 2003). Furthermore, cancer cells themselves have demonstrated overproduction of proinflammatory mediators such as IL-6, stimulating hepatic production of C-reactive protein (CRP) and increasing peripheral blood neutrophil and platelet counts (Matowicka-Karna J, 2013; Ying HQ, 2014; Li Y, 2016). Elevated levels of C-reactive protein, neutrophils and lymphocytes in peripheral blood samples of patients with malignancy have been proposed as prognostic tools, such as the Glasgow Prognostic Score (GPS), neutrophil lymphocyte ratio (NLR) and lymphocyte–monocyte ratio. In this way, a quantitative correlation can be made between blood cell concentrations and patient outcome following surgery.

More recently, a number of papers have pointed specifically to the predictive value of neutrophils in the setting of colorectal liver metastases. In one analysis, Haruki et al failed to demonstrate a correlation between elevated NLR and either disease free, or overall survival. However an independent significant correlation was observed between neutrophilia and poor survival (Haruki K, 2017). Similarly, the significance of neutrophil count on overall survival was underscored by Neal at al., on cox proportional regression analysis (Neal CP, 2011). Analysis of our patient cohort highlighted serum neutrophilia

amongst other factors as an independent predictor of poor survival on multivariate analysis. Systemic inflammation suppresses antitumour immunity by recruiting regulatory T cells and activating pro tumourigenic and pro inflammatory cytokines (Grivennikov SI, 2010). Neutrophils themselves can contribute to continuous angiogenic stimulation by inducing endothelial growth factor (EGFR) release (Taichman NS, 1997). In this way, sustained systemic inflammation may reflect an altered hepatic immune microenvironment, facilitating metastatic success.

Owing to the widely accepted survival advantage conferred following index hepatectomy for CRLM, no randomised controlled trial has been performed to delineate fully the benefit of repeat resection. Notwithstanding the increased technical challenges associated with repeat hepatectomy due to altered hepatic anatomy and intraabdominal adhesions, a number of studies have confirmed its feasibility (Neal CP, 2017; Nanji S, 2017; Hashimoto M, 2016). In contrast to index resections, this current data demonstrates no correlation between preoperative neutrophil lymphocyte ratio and outcomes following repeat resection for recurrent metastatic disease. Analysis of our patient cohort revealed 57 repeat hepatic resections (15% of the total cohort) for recurrent metastasis, a rate which is in keeping with international published data (Lam VW, 2013). Furthermore, 3- and 5- year survival were comparable at 34.8% and 19.7% respectively. There remains ambiguity however, surrounding optimum patient selection for repeat metastatectomy. A recent systematic review identified 22 published series of repeat hepatectomy, with 17 individual prognostic factors, however no one factor was found to be common to all studies. This may in part be due to the small numbers of patients eligible for repeat resection and thus small series size in the published literature. While no one factor was common to all studies, tumour size, margin status and disease-free interval were the most commonly cited prognostic factors amongst published data in that analysis. Similarly,

our analysis also showed increased size of metastasis to be independently associated with poorer overall survival. However, none of the 22 papers examined the role of inflammatory indices as potential prognostic markers of disease outcome.

Despite our finding that elevated neutrophils, and indeed neutrophil lymphocyte ratio were associated with poorer long-term survival following index hepatectomy, this did not translate to the recipients of neoadjuvant chemotherapy or those undergoing repeat resection. Suppan *et al* have previously described a similar phenomenon in breast cancer patients whereby the prognostic value of NLR is negated by the receipt of neoadjuvant chemotherapy. This, they postulate may reflect the effect of systemic chemotherapy on local lymphocytic infiltration of the tumour (Suppan C, 2015). Similarly, those presenting with recurrent disease are more likely to have had multiple cycles of chemotherapy, in itself subverting the systemic immune response. This in turn confounds the reliability of serum inflammatory indices in gauging the prognosis of recurrent disease. While we know the insult of surgery inhibits lymphocyte proliferation causing an immunosuppressive effect systemically (González HD, 2007), little is known of the immune composition of the liver beyond the immediate post-operative period, or indeed whether these changes in immune composition contribute to the increased incidence of metastatic recurrence seen following “curative" hepatectomy. This may reflect alterations in the immune microenvironment at hepatic organ level and warrants further investigation.

Chapter 3

The immune microenvironment of metastatic liver

3.1 Introduction

A favorable microenvironment is critical to the recruitment, development and effector function of resident hepatic immune cell populations. Microenvironmental homeostasis in the liver is maintained by a network of cytokines, chemokines and growth factors (Robinson MW, 2016). However, tumour associated inflammation disrupts this delicate homeostasis which in turn, compromises local tumour surveillance mechanisms (Albillos A, 2014). Molecular cross-talk between tumour-infiltrating immune cells, local tissue-resident immune cell populations, stromal cells and malignant epithelial cells determines the success of metastatic disease (Gajewski TF, 2013). Defining the complex molecular environment of the liver and how it changes with metastases may provide useful prognostic tools, highlighting targets for more effective therapy in metastatic liver disease.

Cytokines and chemokines are increasingly recognized as integral to malignant progression, not only for their ability to induce tumour cell migration but also in their ability to shape an inflammatory niche that is tumour protective (Zhou SL, 2012). Chemokines produced by epithelial cancer cells are known to attract both PMN and lymphocytes to the tumour microenvironment (Fridlender ZG, 2009; Qian BZ, 2010), possessing abilities to modify angiogenesis and tissue remodelling via progenitor cell migration and activation (Anders HJ, 2014). While known to play an active role in the hepatic tumour surveillance mechanism, specific cytokines can accelerate growth in already established hepatic malignancies (Eggert T, 2016). We have previously found increased levels of IL-12 and IL-10 in hepatic parenchyma adjacent to metastatic deposits (Kelly AM, 2004; Kelly AM, 2006) and demonstrated secretion of multiple pro-inflammatory mediators from primary colorectal tumour explants (O'Toole A, 2014;

Michielsen AJ, 2011). In this chapter we extend these observations and present an in-depth analysis of the inflammatory environment of CRLM combined with histological analyses of the PMN and lymphocytic infiltrates in these tissues. We hypothesise that the changed microenvironment present in tumour-bearing liver potentiates ongoing disease by disrupting local hepatic immune activity, acting to attract circulating tumour cells to the liver.

The aim of this chapter was to:

1. Describe the cytokine and immune cell profile of metastatic liver, as distinct from donor healthy liver
2. Establish the effects of metastatic liver cytokine profile on metastatic colorectal cancer cells
3. Ascertain the significance of the altered immune profile of metastatic liver with regards to disease recurrence

3.2 Materials and methods

3.2.1 Study design

In order to characterise the immune cell composition of metastatic liver as it differs from healthy liver and to establish the cytokines are present in metastatic liver, a prospective study of consecutive patients referred for hepatic resection for CRLM to St. Vincent's University Hospital was performed.

3.2.2 Patient recruitment

Patients attending the National Liver Unit at St. Vincent's University Hospital for hepatic metastatectomy secondary to CRC were eligible for inclusion in this study. Eligible patients were approached preoperatively and informed consent was obtained to sample the specimen taken at surgery. Only liver taken as part of the oncological resection was sampled, no additional liver was taken at the time of surgery. Further liver samples were obtained from donor "healthy" livers at the time of organ transplantation. Where donor liver was obtained, informed consent was given by the donor's family prior to sampling. All protocols were approved by the local research and ethics committee and were in accordance with the guidelines of the 1975 Declaration of Helsinki.

3.2.3 Tissue sampling

Fresh tissue was obtained from surgical resection specimens and from donor organs. Biopsies were taken from the tumour, tumour-adjacent macroscopically normal liver, and macroscopically normal liver at the most distant surgical resection margin, (median distance, 40 mm from the metastatic deposit (range 15-96mm). During orthotopic liver transplantation, a wedge biopsy was obtained at the time of implantation of the donor organ. Tissue biopsies were divided in two, one piece was formalin fixed, paraffin

embedded and used for histological analysis; the second piece was placed directly into complete RPMI 1640 medium, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco, Wicklow, Ireland)) and used to generate conditioned media (CM).

3.2.4 Histological analysis; granulocyte and lymphocyte estimation

Routine histological analysis was performed on all specimens from each region of the resected liver (tumour, normal liver tissue both adjacent to and distal from the tumour). Donor liver was assessed in a similar fashion. Formalin fixed paraffin embedded tissue was sectioned and stained with haematoxylin and eosin (H&E). Lymphocytes and polymorphonuclear lymphocytes (PMN) were counted in 10 distinct 40x high power fields (for both lobular and portal regions). The same experienced pathologist (NN) examined all samples.

3.2.5 Generation of tissue CM

Tissue CM was prepared following *ex vivo* culture of biopsies, adapting a previously described protocol (Michielsen AJ, 2011). Briefly, biopsies were cut into equal sized pieces of approximately 5 mm^3 and cultured (in 24-well plates) in complete RPMI 1640 medium. After 72 h in culture, the CM was collected, centrifuged, aliquoted and stored at -20 °C until used for analyses. Donor liver biopsies were cultured in the same fashion and CM samples stored at -20 °C.

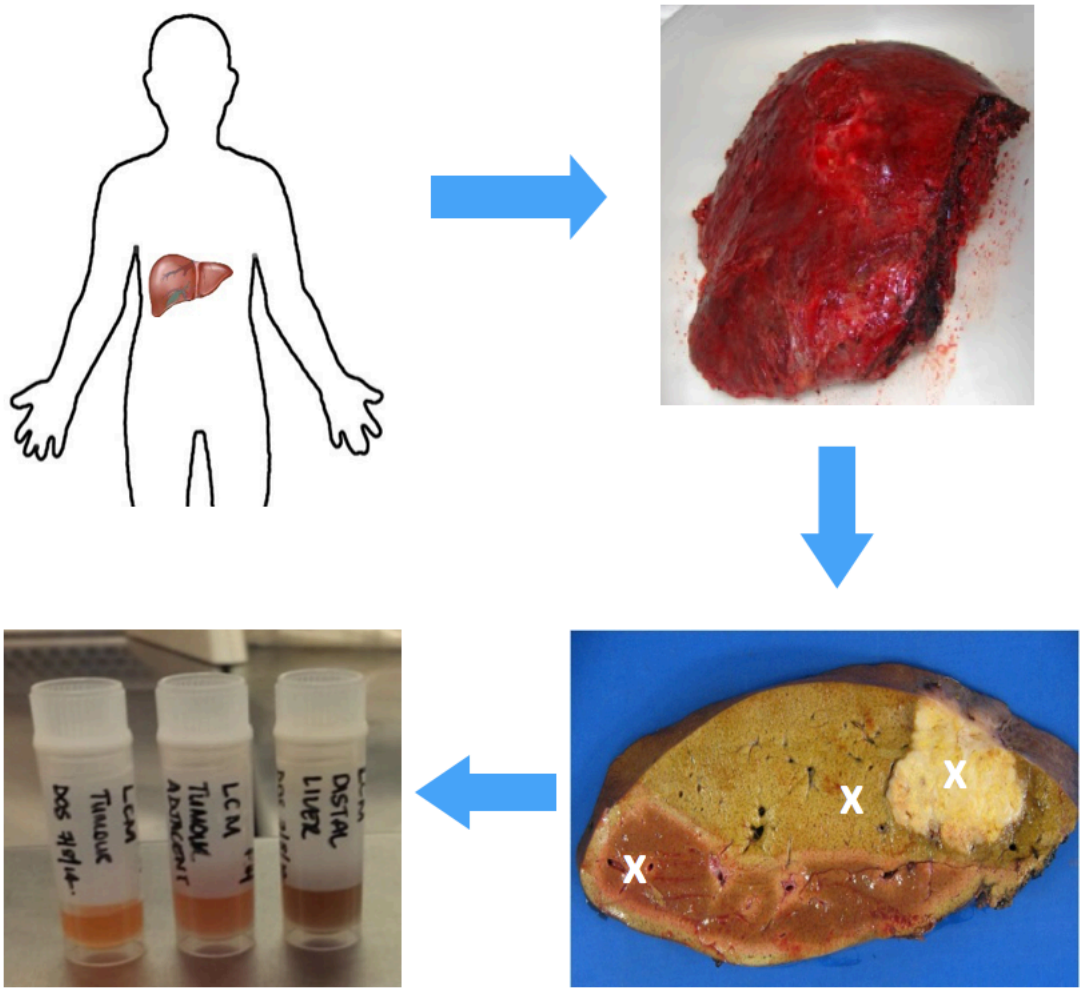


Figure 3.1 Tissue collection and processing. The surgical specimen was resected in the normal fashion, retrieved from the operating room and brought to the pathology laboratory. Samples were obtained from the tumour itself, the hepatic parenchyma adjacent to the tumour and the hepatic parenchyma furthest from the tumour. These samples were then returned to the laboratory to be processed.

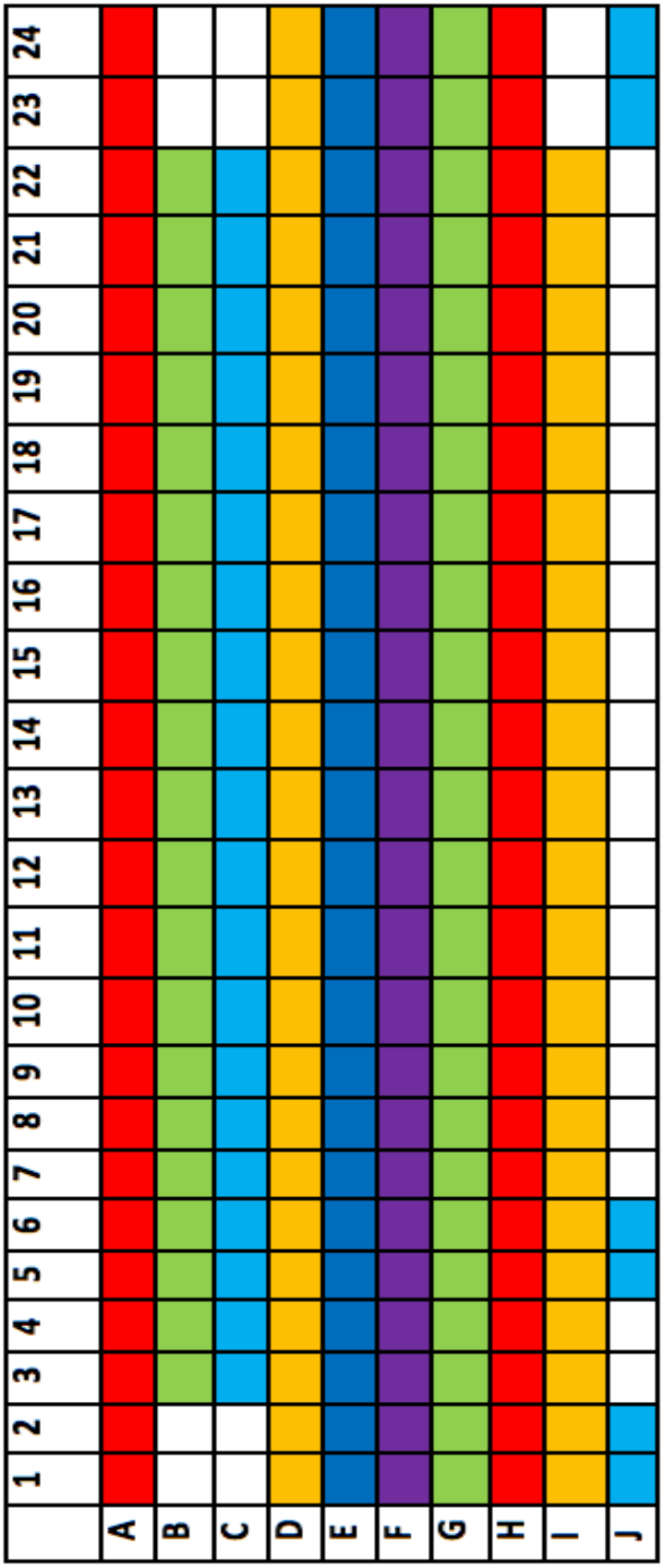


Figure 3.2 Human XL cytokine array coordinates

Table 3.1 Coordinates of each analyte for human XL cytokine array

Coordinate	Analyte/Control	Entrez Gene ID	Alternative Nomenclature
A1, A2	Reference Spots	N/A	RS
A3, A4	Adiponectin	9370	Acrp30
A5, A6	Aggrecan	176	Aggrecan 1
A7, A8	Angiogenin	283	-
A9, A10	Angiopoietin-1	284	Ang-1, ANGPT1
A11, A12	Angiopoietin-2	285	Ang-2, ANGPT2
A13, A14	BAFF	10673	BLyS, TNFS13B
A15, A16	BDNF	627	Brain-derived Neurotrophic Factor
A17, A18	Complement Component C5/C5a	727	C5/C5a
A19, A20	CD14	929	-
A21, A22	CD30	943	TNFRSF8
A23, A24	Reference Spots	N/A	RS
B3, B4	CD40 Ligand	959	CD40L,TNFSF5, CD154, TRAP
B5, B6	Chitinase 3-like 1	1116	CHI3L1, YKL-40
B7, B8	Complement Factor D	1675	Adipsin, CFD
B9, B10	C-reactive Protein	1401	CRP
B11, B12	Crypto-1	6997	Teratocarcinoma-derived Growth Factor
B13, B14	Cystatin C	1471	CST3, ARMD11
B15, B16	Dkk-1	22943	Dickkopf-1
B17, B18	DPPIV	1803	CD26, DPP4, Dipeptidyl-pepidase IV
B19, B20	EGF	1950	Epidermal Growth Factor
B21, B22	Emmprin	682	CD147, Basigin
C3, C4	ENA-78	6374	CXCL5
C5, C6	Endoglin	2022	CD105, ENG
C7, C8	Fas Ligand	356	TNFSF6, CD178, CD95L
C9,C10	FGF basic	2247	FGF-2
C11, C12	FGF-7	2252	KGF
C13, C14	FGF-19	9965	-
C15, C16	Flt-3 Ligand	2323	FLT3LG
C17, C18	G-CSF	1440	CSF3
C19, C20	GDF-15	9518	MIC-1
C21, C22	GM-CSF	1437	CSF2
D1, D2	GRO-α	2919	CXCL1, MSGA-α
D3, D4	Growth Hormone	2688	GH, Somatotropin
D5, D6	HGF	3082	Scatter Factor, SF
D7, D8	ICAM-1	3383	CD54

Coordinate	Analyte/Control	Entrez Gene ID	Alternative Nomenclature
D9, D10	IFN-γ	3458	IFNG
D11, D12	IGFBP-2	3485	-
D13, D14	IGFBP-3	3486	-
D15, D16	IL-1α	3552	IL-1F1
D17, D18	IL-1β	3553	IL-1F2
D19, D20	IL-1rα	3557	IL-1F3
D21, D22	IL-2	3558	-
D23, D24	IL-3	3562	-
E1, E2	IL-4	3565	-
E3, E4	IL-5	3567	-
E5, E6	IL-6	3569	-
E7, E8	IL-8	3576	CXCL8
E9, E10	IL-10	3586	-
E11, E12	IL-11	3589	-
E13, E14	IL-12p70	3593	-
E15, E16	IL-13	3596	-
E17, E18	IL-15	3600	-
E19, E20	IL-16	3603	-
E21, E22	IL-17A	3605	IL-17, CTLAB
E23, E24	IL-18 BPa	10068	-
F1, F2	IL-19	29949	-
F3, F4	IL-22	50616	IL-TIF
F5, F6	IL-23	51561	IL-23A, SGRF
F7, F8	IL-24	3627	C49A, FISP, MDA-7, MOB-5, ST16
F9, F10	IL-27	246778	-
F11, F12	IL-31	386653	-
F13, F14	IL-32α/β/γ	9235	-
F15, F16	IL-33	90865	C9orf26, DVS27, NF-HEV
F17, F18	IL-34	146433	C16orf77
F19, F20	IP-10	3627	CXCL10
F21, F22	I-TAC	6373	CXCL11, SCYB9B
F23, F24	Kallekrein 3	354	PSA, KLK3
G1, G2	Leptin	3952	OB
G3, G4	LIF	3976	-
G5, G6	Lipocalin-2	3934	NGAL, LCN2, Siderocalin
G7, G8	MCP-1	6347	CCL2, MCAF
G9, G10	MCP-3	6354	CCL7, MARC
G11, G12	M-CSF	1435	CSF-1
G13, G14	MIF	4282	-

Coordinate	Analyte/Control	Entrez Gene ID	Alternative Nomenclature
G15, G16	MIG	4283	CXCL9
G17, G18	MIP-1α/MIP-1β	6348/6351	CCL3/CCL4
G19, G20	MIP-3α	6364	CCL20, Exodus-1, LARC
G21, G22	MIP-3β	6363	CCL19, ELC
G23, G24	MMP-9	4318	CLG4B, Gelatinase B
H1, H2	Myeloperoxidase	4353	MPO, Lactoperoxidase
H3, H4	Osteopontin	6696	OPN
H5, H6	PDGF-AA	5154	-
H7, H8	PDGF-AB/BB	5154/5155	-
H9, H10	Pentraxin-3	5806	PTX3, TSG-14
H11, H12	PF4	5196	CXCL4
H13, H14	RAGE	177	-
H15, H16	RANTES	6352	CCL5
H17, H18	RBP4	5950	-
H19, H20	Relaxin-2	6019	RLN2, RLXH2
H21, H22	Resistin	56729	ADSF, FIZZ3, RETN
H23, H24	SDF-1α	6387	CXCL12, PBSF
I1, I2	Serpin E1	5054	PAI-I, PAI-1, Nexin
I3, I4	SHBG	6462	ABP
I5, I6	ST2	9173	IL-1R4, IL1RL1, ST2L
I7, I8	TARC	6361	CCL17
I9, I10	TFF3	7033	ITF, TFI
11, I12	TfR	7037	CD71, TFR1, TFRC, TRFR
I13, I14	TGF-α	7039	TGFA
I15, I16	Thrombospondin	7057	THBS1, TSP-1
I17, I18	TNF-alpha	7124	TNFSF1A
I19, I20	uPAR	5329	PLAUR
I21, I22	VEGF	7422	BEGFA
J1, J2	Reference Spots	N/A	RS
J5, J6	Vitamin D BP	2638	VDB, DBP, VDBP
J23, J24	Negative Controls	N/A	Control (-)

3.2.6 Analysis of inflammatory mediators

An initial screen of cytokines present in liver CM was performed using the Proteome Profiler Human XL Cytokine Array (R&D Systems; Abingdon UK). Samples of CM from two metastatic livers and two donor livers were analysed according to manufacturer's instructions. Densitometry analysis of the array was performed using ImageJ software (U.S. National Institute of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). Raw signal intensity was normalized to background from negative control spots. The mean signal of each technical replicate was displayed as a heatmap (R studio, Version 1.0.136 & R Version 3.2.1).

3.2.7 Measurement of cytokine levels in liver conditioned media

Levels of CXCL5, IL-6, CXCL1, VEGF, GMCSF, LIF, CCL3/4, G-CSF, CCL5, CXCL10, CCL2 and IL-8 protein were determined in CM from 25 metastatic livers using the Milliplex Map Kit Human Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore). Fluorescent signals were measured with a Luminex 200 reader (Luminex Corp., Austin, TX). Data were calculated by generating a calibration curve obtained using recombinant cytokines specified above. Concentrations of cytokines were calculated using a five-parameter logistic curve-fitting method. Cytokine and chemokine concentrations in all samples were then normalized to the total protein content determined for each sample using a BCA assay (Pierce, Fischer Scientific, Dublin 15, Ireland).

3.2.8 Effect of tissue CM on chemotaxis of metastatic cells

Metastatic CRC (SW620) cells were seeded at a density of 2.5 $x10^4$ cells in the upper compartment of Matrigel-coated inserts (8-mm pore size; BioCoat, BD Biosciences,

Erembodegem- Dorp, Belgium) in serum-free medium. Liver-conditioned medium was used as chemoattractant in the lower chamber. After 48 hours of incubation, cells remaining in the upper chamber were removed with a PBS-soaked cotton swab. Cells on the lower face of the chambers were fixed using 1% glutaraldehyde, stained with 0.1% crystal violet, and imaged under a light microscope at a magnification of 10x, averaging three random fields per insert. Crystal violet staining was quantified using ImageJ software, and cell invasion was expressed as percentage compared to control inserts with no chemoattractant.

Table 3.2 Detection range for each analyte analysed by multiplex magnetic bead panel

	Analyte	Detection Range	Volume per well
1	ENA 78 (CXCL5)	15.6-1,000 pg/ml	25µl
2	LIF	1.0-100,000 pg/ml	25µl
3	G-CSF	31.2-2,000 pg/ml	25µl
4	GM-CSF	15.6-1,000 pg/ml	25µl
5	GRO (CXCL1)	31.2-2,000 pg/ml	25µl
6	IL6	3.2-10,000 pg/ml	25µl
7	IL8	3.2-2,000 pg/ml	25µl
8	IP10 (CXCL10)	31.2-2,000 pg/ml	25µl
9	MCP-1(CCL2)	15.6-1,000 pg/ml	25µl
10	RANTES (CCL5)	15.6-1,000 pg/ml	25µl
11	VEGF	31.2-2,000 pg/ml	25µl
12	MIP-1α (CCL3)	7.81-500pg/ml	25µl

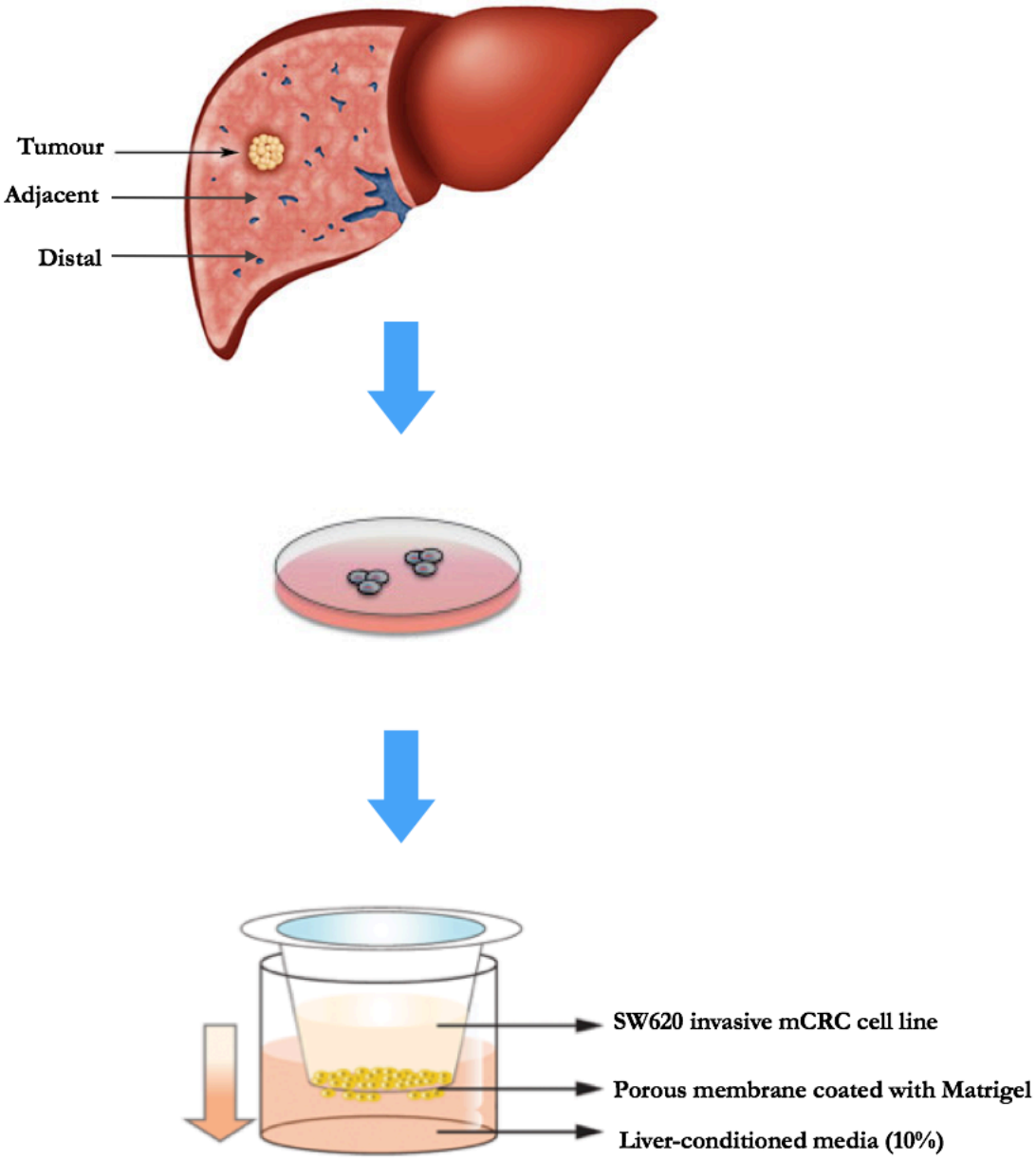


Figure 3.3 Chemotaxis assay. Conditioned media is obtained as previously described and placed at the base of each well. SW620 metastatic colorectal cells are placed in the well inserts and separated from the conditioned media by a permeable membrane coated with matrigel. After incubation, malignant colorectal cells are stained to ascertain if migration through the porous membrane has occurred.

3.2.9 Statistical analysis

Univariate and multivariate logistic regression analyses were used to examine the effect of variables on long term mortality, postoperatively. Univariate associations with long-term survival were determined using univariate Cox regression analysis, Kaplan-Meier analysis, and the log-rank test. Multivariable analyses were performed by Cox proportional hazards regression analysis (using a stepwise backward procedure), incorporating all variables with $P < .10$ on univariate analysis. Statistical significance was defined as $P < .05$. These analyses were conducted using a software program (SPSS version 16.0; SPSS, Inc, Chicago, Illinois).

Statistical analyses of immune cell frequencies in donor and metastatic liver were carried out using Mann-Whitney U test (GraphPad Prism software, version 6.0, La Jolla, CA, USA). Kaplan Meier survival curves for time to recurrence were generated for patients who had neutrophil counts <4 (N=13) and >4 (N=11) (SPSS, Version 21.0 (2012), IBM Corp, New York, USA). Levels of cytokines present within different regions of metastatic liver were compared using one-way ANOVA with Tukey's multiple comparison test (Graph Pad Software, version 6.0*)*. Pearson correlations were calculated using SPSS and data inputted at https: discover.nci.nih.gov/cimminer/ to draw correlation matrices.

3.3 Results

Twenty-five patients undergoing partial hepatectomy for CRLM were recruited to this arm of the study. Median patient age was 64 years with 15 male participants and 10 female. Of the patients included, 19 in total received neoadjuvant chemotherapy, just 10 of whom showed evidence of treatment response on histological analysis. The vast majority of metastases sampled were consistent with moderately differentiated adenocarcinoma of colonic origin (n=20). Full particulars of patient variables are detailed in Table 3.3.

3.3.1 Distal metastatic liver differs from healthy liver on histological analysis

Each metastatic liver was stained with haematoxylin and eosin (H&E) and sections of macroscopically normal liver, distal from the tumour deposit, in CRLM cases (n=25) and liver donor biopsy controls (n=13) were examined. Lymphocytes and PMN were counted in 10 separate 40x high power fields per slide for both lobular and portal areas (representative images shown in Figures 3.4 and 3.5) of distal metastatic liver and donor liver. Lymphocytes and PMN were found in all tissue sections examined. Significant inter-individual variation was noted in both the PMN and lymphocyte counts in the periportal and lobular regions when healthy liver was compared to distal metastatic liver (Figure 3.6). Lymphocyte counts were significantly increased in the periportal and lobular regions of metastatic liver when compared with healthy donor tissue. Numbers of PMNs were similar in the parenchyma of healthy and metastatic liver tissue; however, they were significantly decreased in the peri-portal regions of metastatic liver when compared with healthy donor tissue. When these results were expressed as ratios (neutrophil: lymphocyte, NLR), even more dramatic differences were observed between healthy and tumour bearing liver (Figure 3.6)

Table 3.3 Characteristics of prospective patient cohort

Demographics	
Number of patients	25
Median patient age, yr (range)	64 (39-86)
Male/female	15/10
Preoperative chemotherapy (n)	
- **No treatment**	6
- **Treatment partial response**	10
- **Treatment no response**	9
Disease Burden	
- **Number of metastases, median (range)**	2 (1-10)
- **Median tumour size, mm (range)**	32 (2-420)
- **Satellite lesions (n)**	4
- **Lymphovascular invasion (n)**	6
Tumour Biology (n)	
- **Mucinous Adenocarcinoma**	3
- **Poorly Differentiated Adenocarcinoma**	2
- **Moderately Differentiated Adenocarcinoma**	20
Histology of background liver (n)	
- **Normal**	8
- **Steatosis**	16
Fibrosis	1

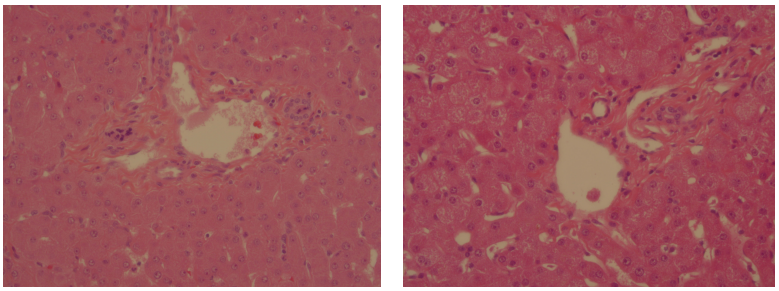


Figure 3.4 Representative histological photographs of haematoxylin and eosin (H&E) stained sections of normal healthy liver, samples of which were obtained from donor liver at transplantation. Lymphocytes and neutrophils were counted in 10 separate 40x high power fields per slide for both lobular and portal areas of each sample.

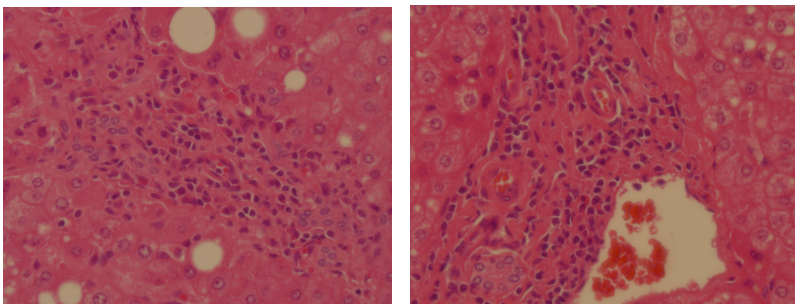


Figure 3.5 Representative photographs of haematoxylin and eosin (H&E) stained sections of metastatic liver, taken distal to the tumour deposit. Lymphocytes and neutrophils were counted in 10 separate 40x high power fields per slide for both lobular and portal areas of each sample.

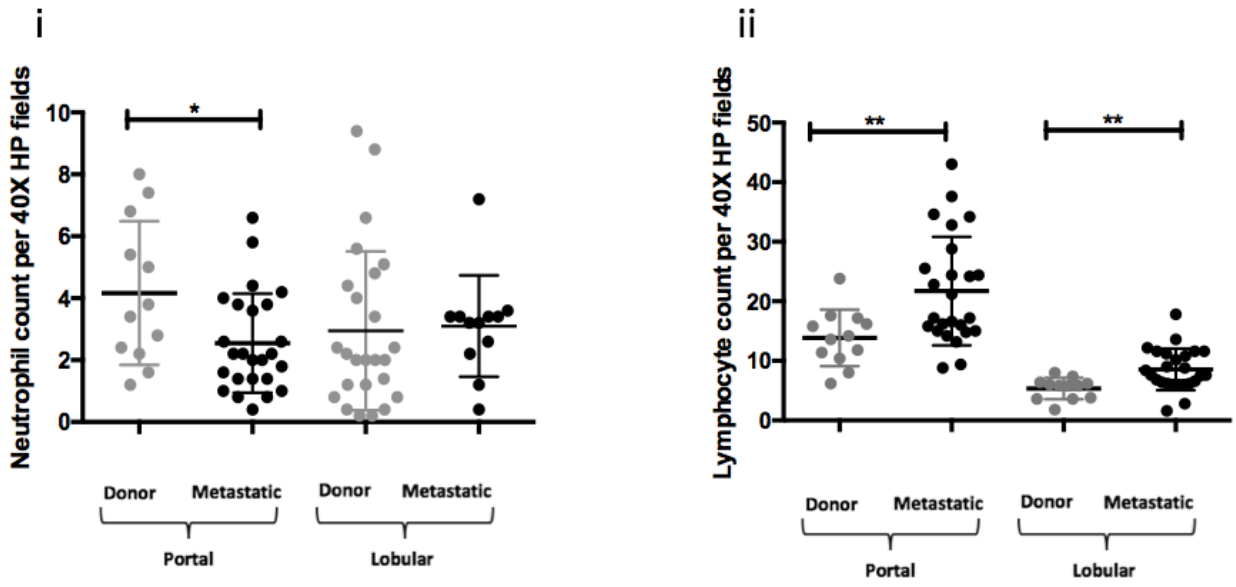


Figure 3.6 Quantification of neutrophils and lymphocytes in metastatic and donor liver. Neutrophil counts (i) and lymphocyte counts (ii) displayed significant differences between diseased and healthy liver for both portal and lobular areas. Results were analysed with Mann Whitney U test, *p<0.05; **p <0.01 ***p <0.001.

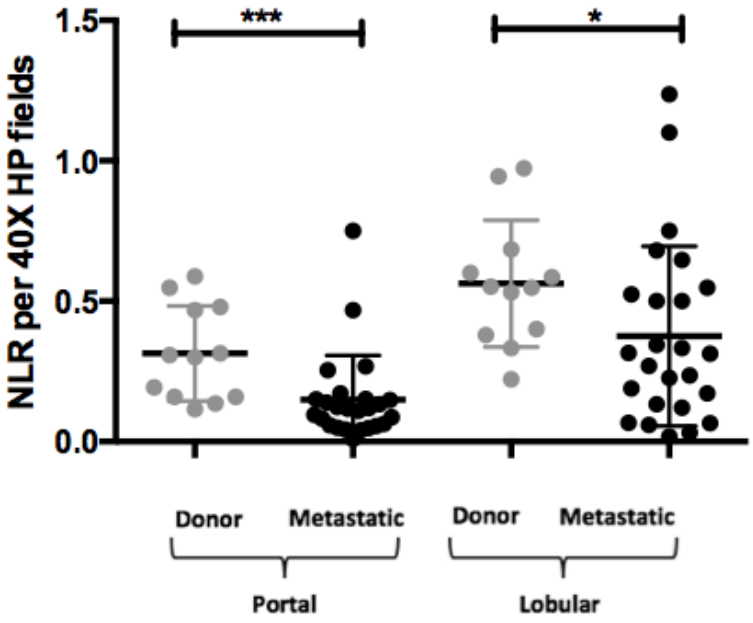


Figure 3.7 Quantification of NLR in metastatic and donor liver. When expressed as a ratio, again significant differences were seen between the immune cell distribution of metastatic and distal liver for both portal and lobular regions. Results were analysed with Mann Whitney U test, *p<0.05; **p <0.01 ***p <0.001.

3.3.2 Hepatic PMN counts correlate with metastatic recurrence following curative hepatectomy

On follow up of our patient cohort over a median of 18 months, 52% (13 of 25) developed recurrent hepatic metastasis. Patients who demonstrated recurrence had fewer neutrophils at the distal resection margins, both in portal and lobular regions than patients who remained in remission (Figure 3.8(i)). These patients also had significantly increased lymphocyte counts in both regions (Figure 3.8 (ii)). Depleted neutrophil counts (<4/High Powered Field (HPF)) at the distal resection margin were associated with hepatic metastatic recurrence ($p=0.015$). Furthermore, patients with neutropenic hepatic parenchyma had a significantly shorter disease-free survival ($p=0.028$) (Fig 3.9)

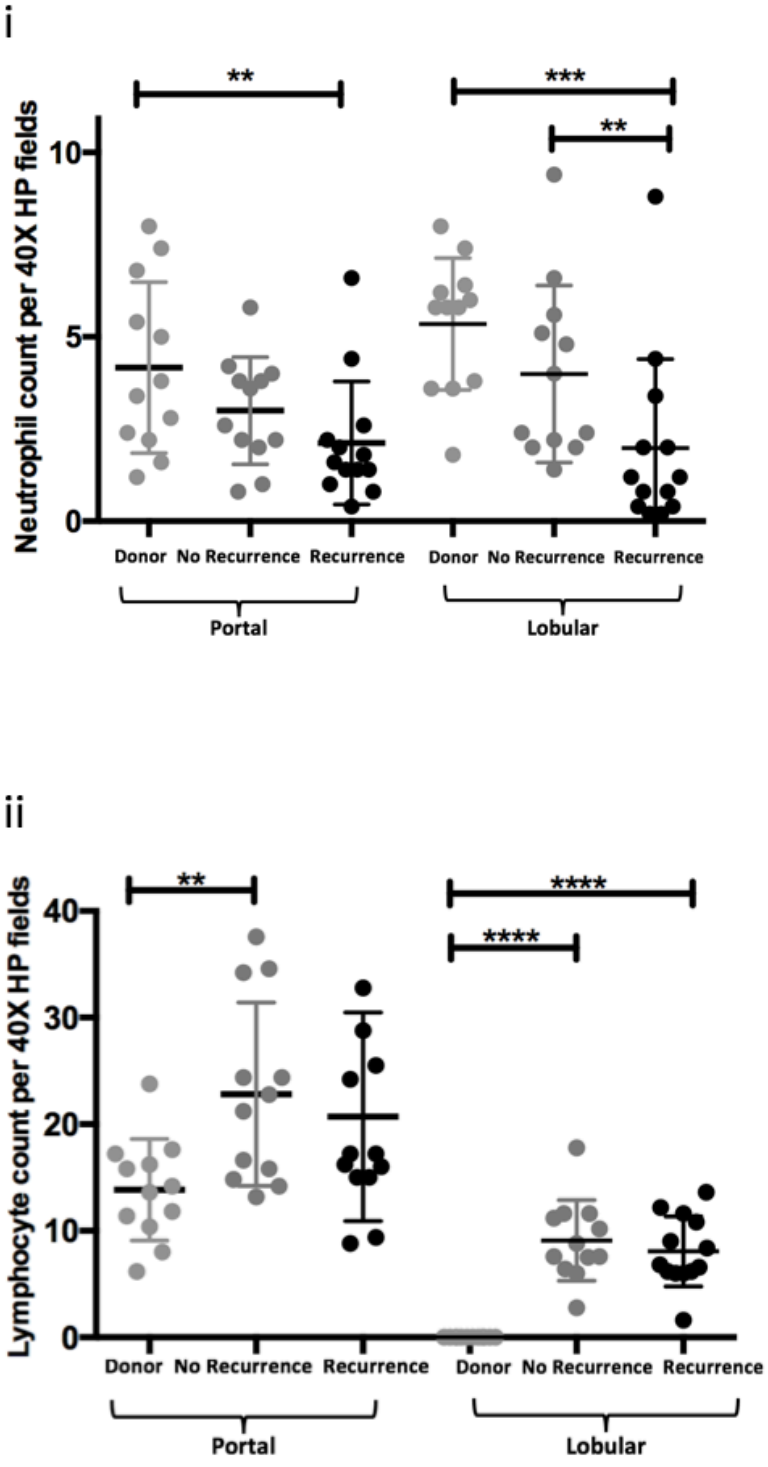


Figure 3.8 Hepatic neutropenia correlates with metastatic recurrence. Neutrophil counts (i) and Lymphocyte counts (ii) comparing donor liver, distal metastatic liver of patients that remained disease free and distal metastatic liver of patients that recurred stained with haematoxylin-eosin; 40 x magnification. Results were analysed with Mann Whitney U test, *p<0.05; **p <0.01 ***p <0.001 ****p<0.0001

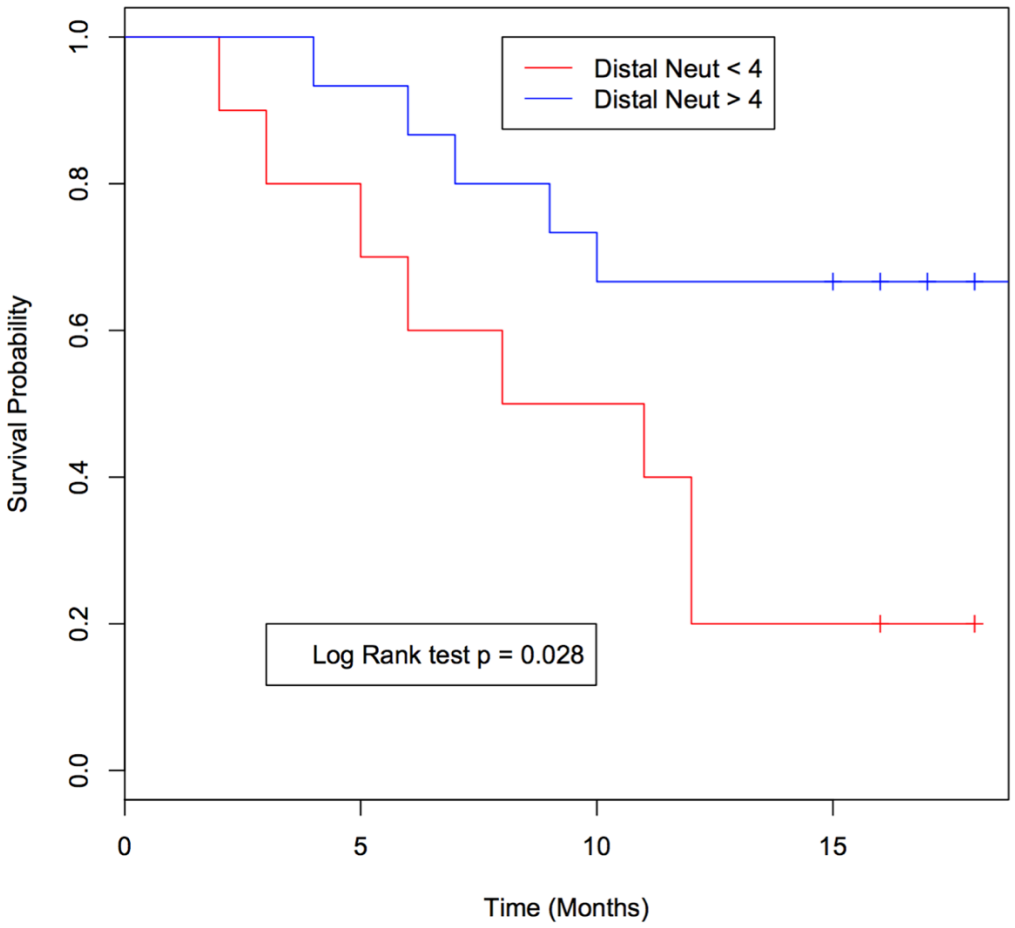


Figure 3.9 Hepatic neutropenia correlates with time to recurrence. Kaplan-Meier curve illustrating time to recurrence in both neutropenic group (neutrophils <4) and neutrophilic group (neutrophils >4), a significant difference was calculated using log-rank test p=0.028

3.3.3 The cytokine profile of distal metastatic liver differs from healthy liver

The cytokine profile of the CM from 2 donor livers were analysed by protein array (Figure 3.9). Of 102 cytokines measured, 25 were highly expressed in donor liver CM (Figure 3.10). These included cytokines of hepatic origin such as retinol binding protein, platelet factor 4 and C-reactive protein. Other inflammatory mediators including: CCL5, CCL2, IL-8 and ICAM-1, resistin, lipocalin, macrophage migration inhibitory factor (MIF), and Dipeptidyl peptidase-4 (DPP4) were also highly expressed. This suggests the presence of a constant state of dynamic benign inflammation in the healthy hepatic microenvironment.

Next, we repeated this analysis using CM generated from livers bearing CRLM (n=2). Biopsies from the tumour, normal tissue adjacent to or distal from the metastatic deposit were cultured *ex vivo* and CM collected. Eight cytokines; CXCL5, IL-6, CXCL1, VEGF, GM-CSF, LIF, G-CSF and CCL3 were elevated in CM obtained from normal liver tissue distal from the metastatic deposit compared to donor liver CM (Fig 2B). While the distal and adjacent tissue CM from patient 1 contained CCL3/CCL4, that of patient 2 did not. Lower levels of CCL5 were seen in tumour CM of both patients when compared to CM from adjacent and distal metastatic liver and indeed, donor controls. The converse was observed with CXCL10, where lower levels were demonstrated in CM of donor and distal metastatic liver but elevated in tumour. Data presented as a heat map (Figure 3.10) shows the cytokine profile of both donor livers clustered together despite demographic dissimilarity in both patients. Furthermore, both tumour CM exhibited similar cytokine profiles and thus clustered together. However, this pattern was not observed with adjacent or distal CM suggesting that whilst a similar cytokine signature exists in the tumour microenvironment, increased inter-individual variation exists in the cytokine profile of parenchyma adjacent and distal to metastases.

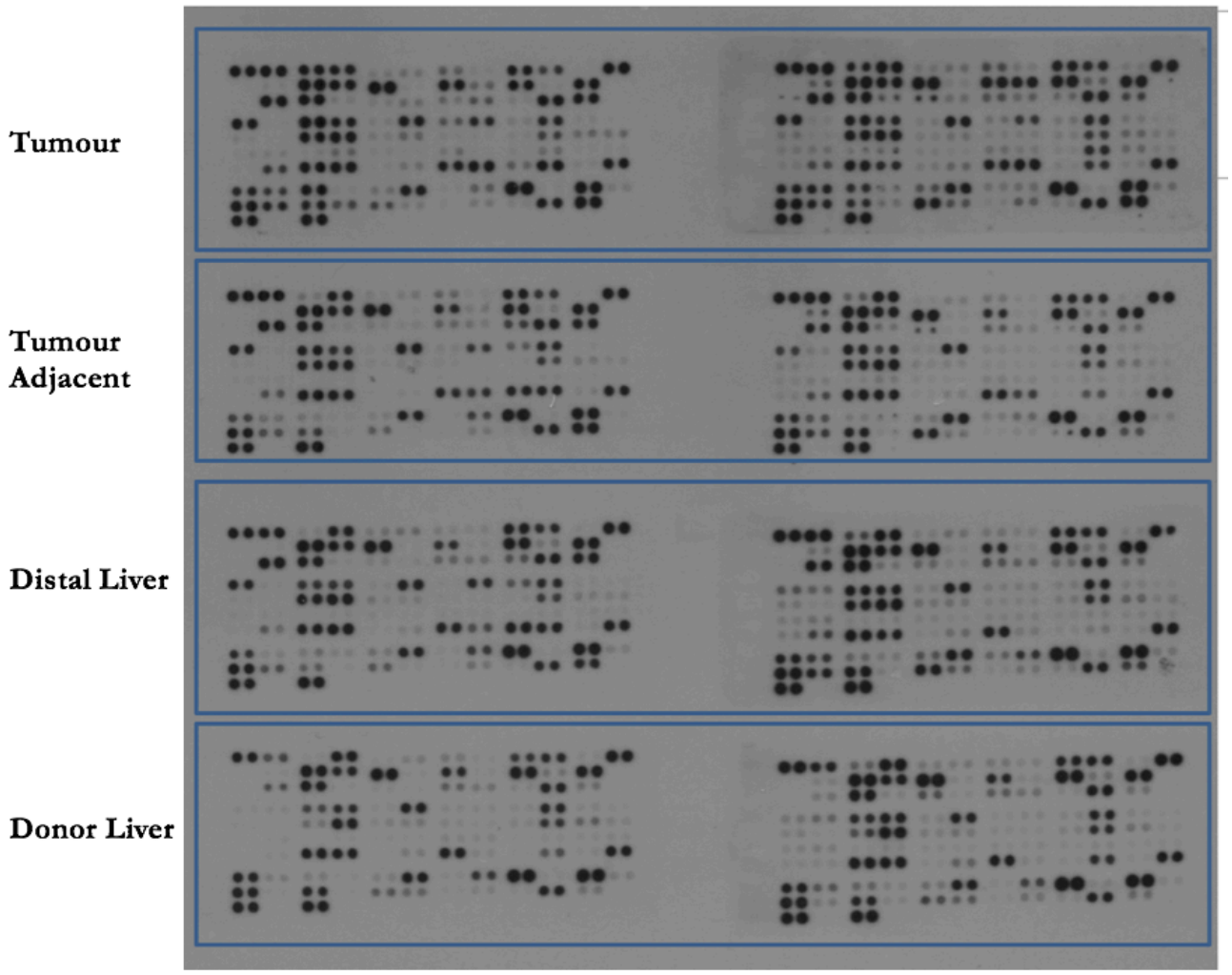


Figure 3.10 Protein array of cytokine prevalence in conditioned media of 2 nominal donor liver samples and 3 distinct areas of 2 metastatic livers.

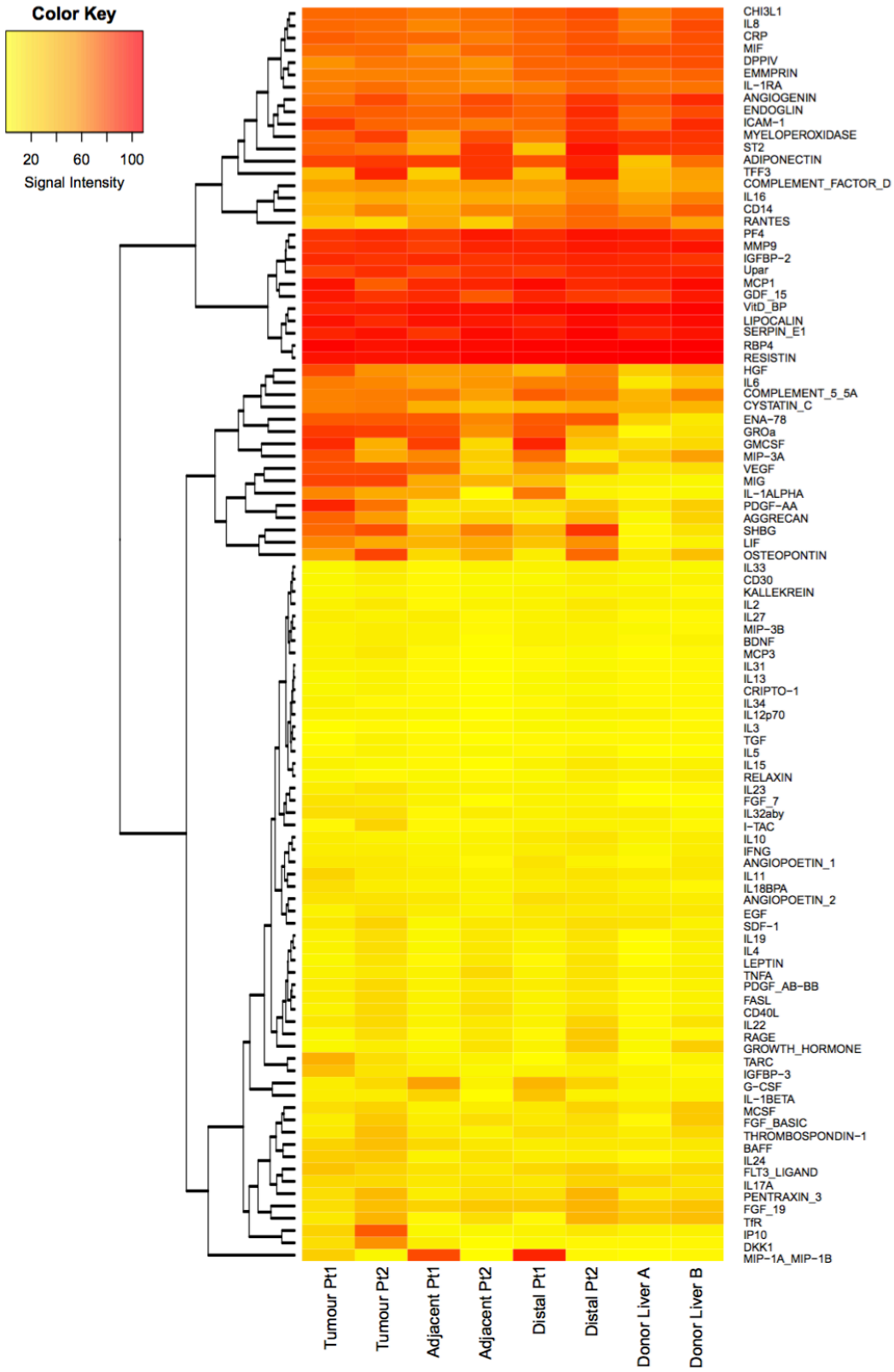


Figure 3.11 Heat map illustration of protein array cytokine analysis. Abundant cytokines illustrated in red with lower expressed cytokines illustrated in yellow.

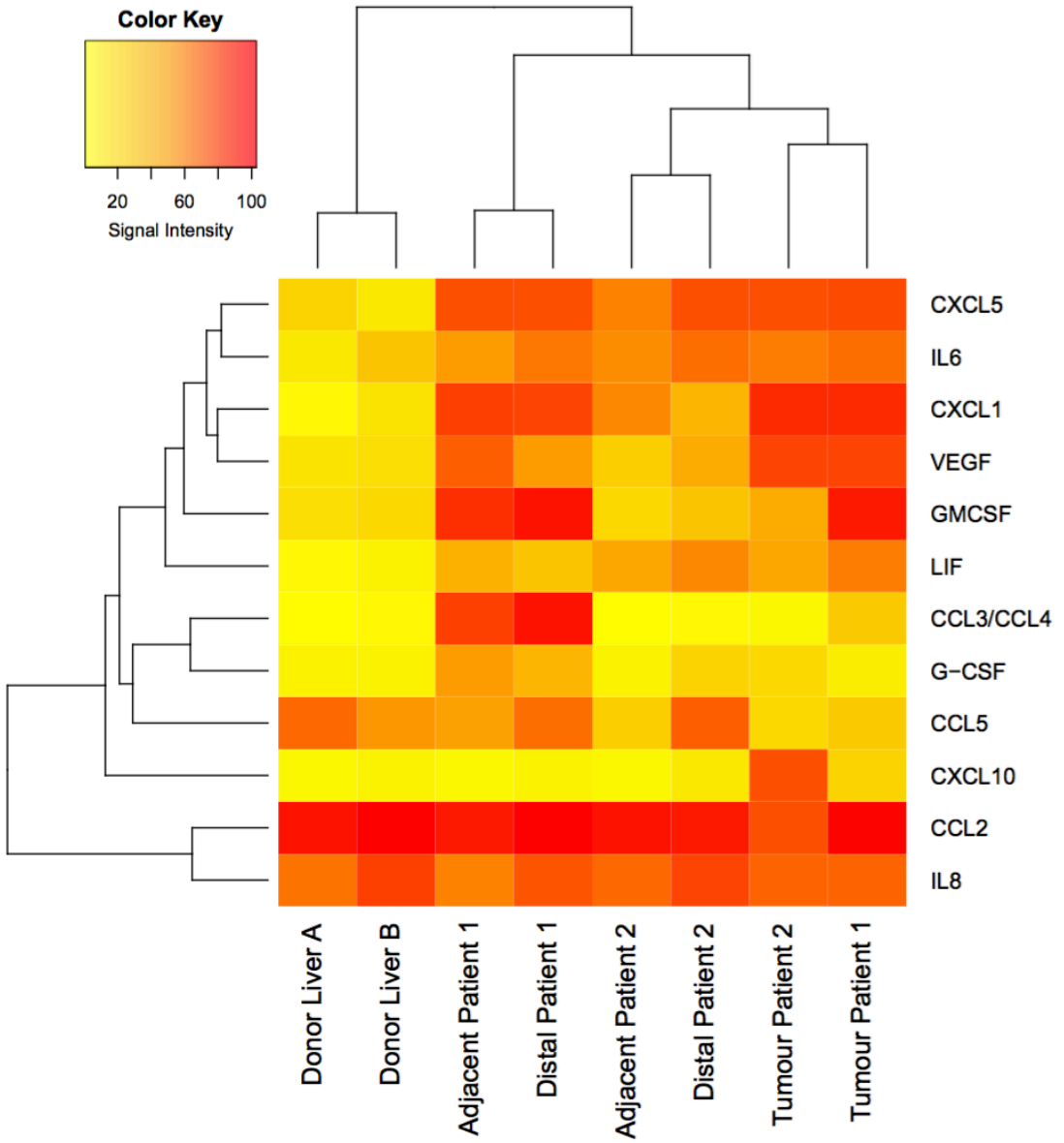


Figure 3.12 The twelve most differentially expressed cytokines between donor (n=2) and metastatic (n=2) liver.

3.3.4 The hepatic cytokine milieu is perturbed in the presence of metastasis

The cytokine profile of tissue CM from 3 distinct areas (tumour tissue, tumour adjacent normal tissue and normal liver tissue at the resection margin) of 25 metastatic livers were quantified by multiplex ELISA. Correlation matrices summarizing the degree of co-expression of inflammatory mediators at each site (i) distal from the tumour, (ii) adjacent to the tumour and (iii) tumour are shown in Figure 3.12. CXCL5 and CXCL10 display the weakest correlation with other cytokines across all tissue sites. LIF and VEGF correlate weakly in the distal tissue but show a strong correlation in tumour.

Graphical representation for each of the 12 cytokines are shown in Figures 3.13 - 3.18 with differing concentration levels shown at each area of liver sampled. While minimal concentrations of LIF are seen in healthy donor liver, significant concentrations are seen in all areas of metastatic liver. An interleukin 6 class cytokine, LIF affects cell growth by inhibiting terminal differentiation in myeloid cells. Conversely, high concentrations of IL6 is concentrated in the tumour when compared to other areas of metastatic liver. Elevated concentrations of GM-CSF also a neutrophil stimulant were seen across metastatic liver, and significantly higher than concentrations seen in donor liver. While wide variability was noted for each patient sample in concentrations of MCP-1 across metastatic liver, concentrations of ENA 78 and IL8 both neutrophil chemoattractants were not significantly different between healthy and diseased samples. VEGF, in a similar pattern to IL6 was concentrated in the tumour itself with lower concentrations seen distal to the tumour. Significant concentrations of IP10, a lymphocyte chemoattractant was seen in tumour media and in distal metastatic liver when compared to adjacent and donor liver. Inversely, higher concentrations of RANTES a monocyte chemoattractant were seen in donor liver when compared to tumour conditioned media.

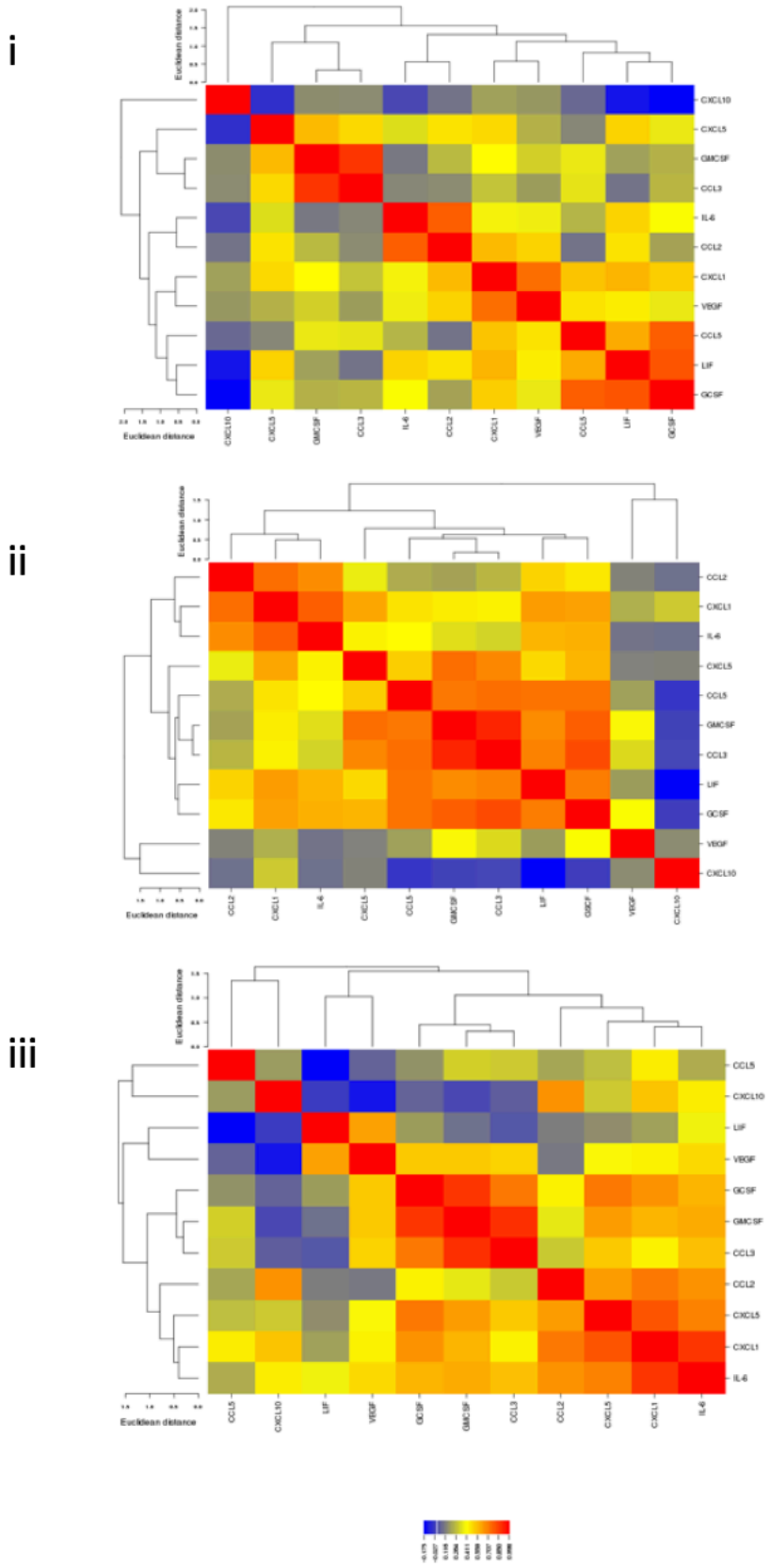


Figure 3.13 Correlation heat maps illustrating cytokine co-expression in (A) tumour, (B) normal liver adjacent to the tumour and (C) normal liver distal to the tumour

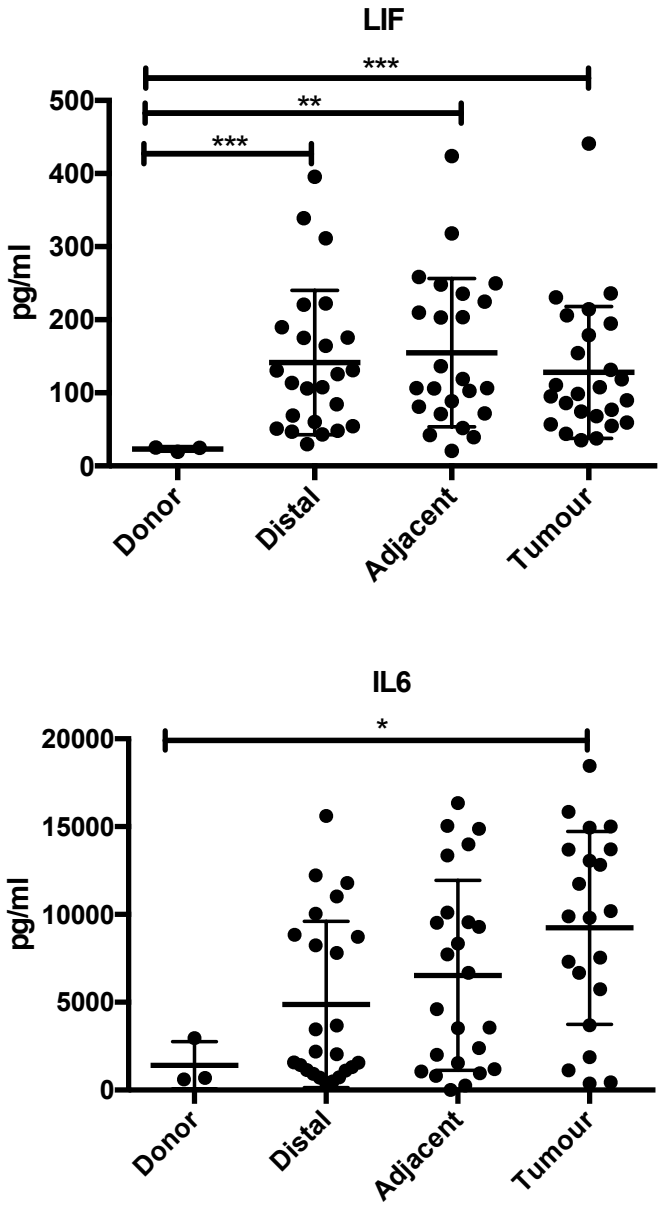


Figure 3.14 Levels of LIF and IL6 in donor, distal metastatic liver, liver adjacent to tumour and tumour conditioned media. Cytokines were quantified by Multiplex ELISA in order to establish levels of expression across metastatic liver. Results were analysed using ANOVA with Tukey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001 ****p<0.0001

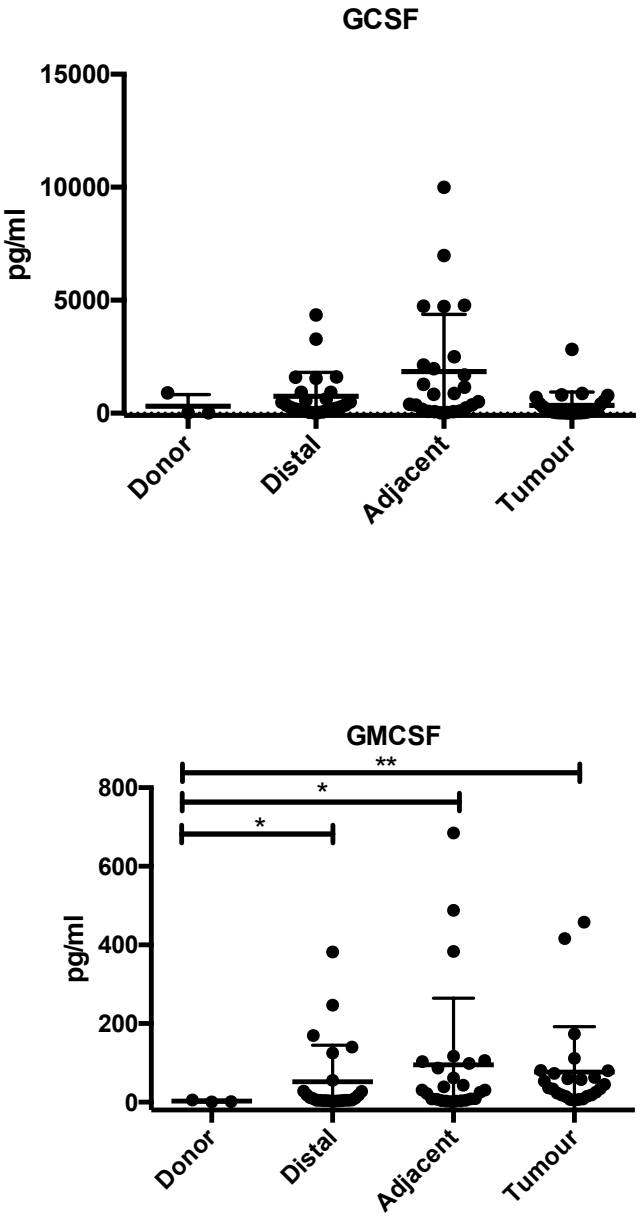


Figure 3.15 Levels of GCSF and GM-CSF in donor, distal metastatic liver, liver adjacent to tumour and tumour conditioned media. Cytokines were quantified by Multiplex ELISA in order to establish levels of expression across metastatic liver. Results were analysed using ANOVA with Tukey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001.

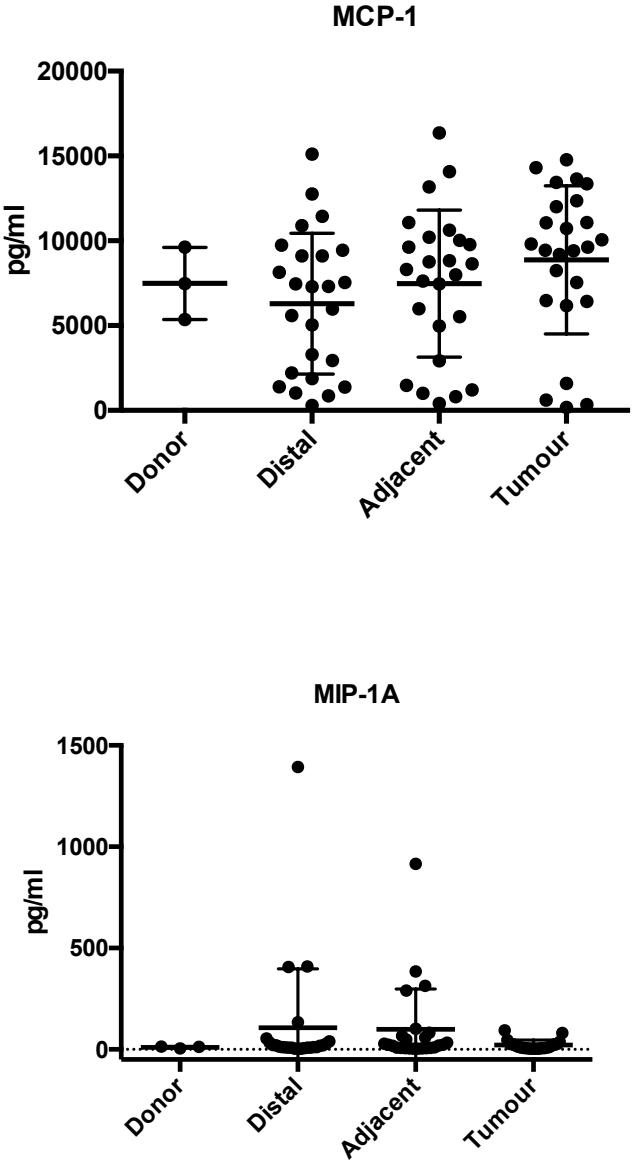


Figure 3.16 Levels of MCP-1 and MIP-1A in donor, distal metastatic liver, adjacent metastatic and tumour conditioned media. Cytokines were quantified by Multiplex ELISA in order to establish levels of expression across metastatic liver. Results were analysed using ANOVA with Tukey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001.

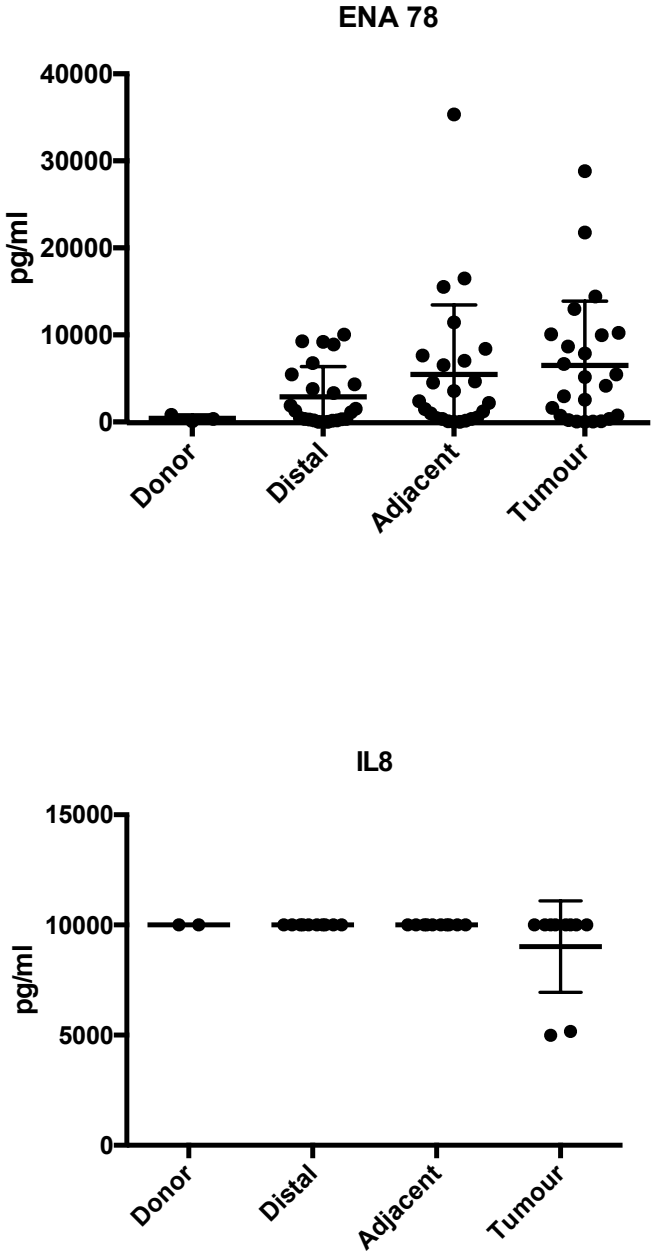


Figure 3.17 Levels of ENA 78 and IL8 in donor, distal metastatic liver, adjacent metastatic and tumour conditioned media. Cytokines were quantified by Multiplex ELISA in order to establish levels of expression across metastatic liver. Results were analysed using ANOVA with Tukey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001.

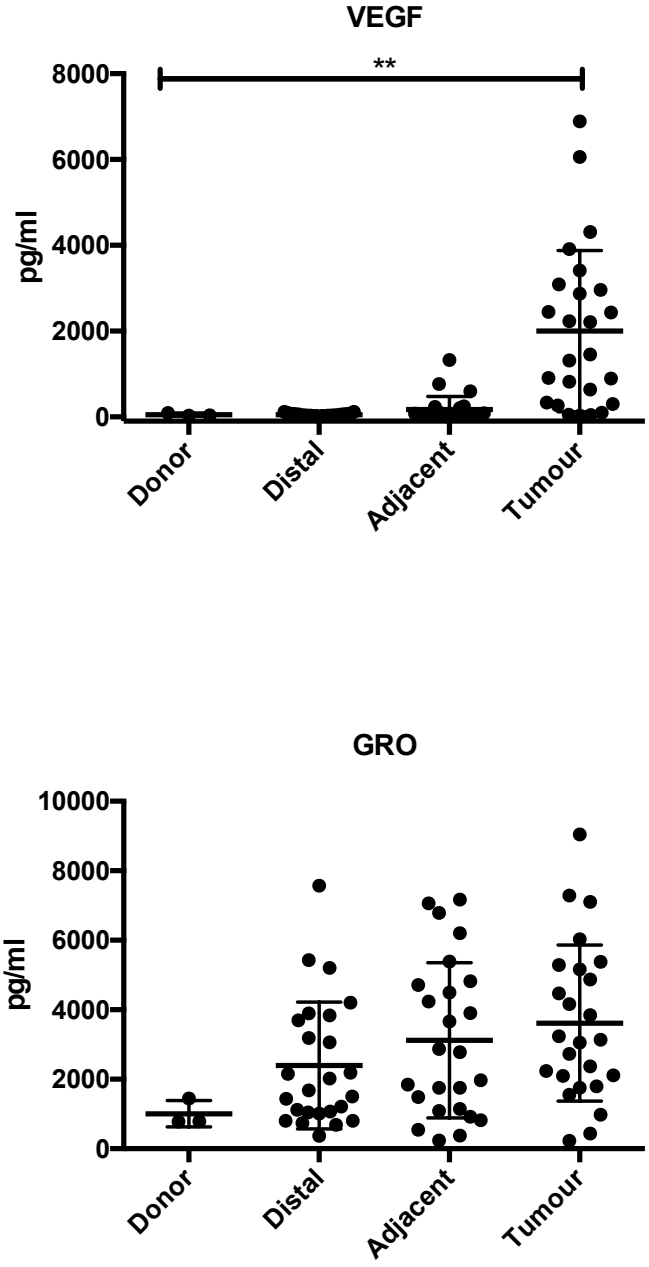


Figure 3.18 Levels of VEGF and GRO in donor, distal metastatic liver, adjacent metastatic and tumour conditioned media. Cytokines were quantified by Multiplex ELISA in order to establish levels of expression across metastatic liver. Results were analysed using ANOVA with Tukey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001.

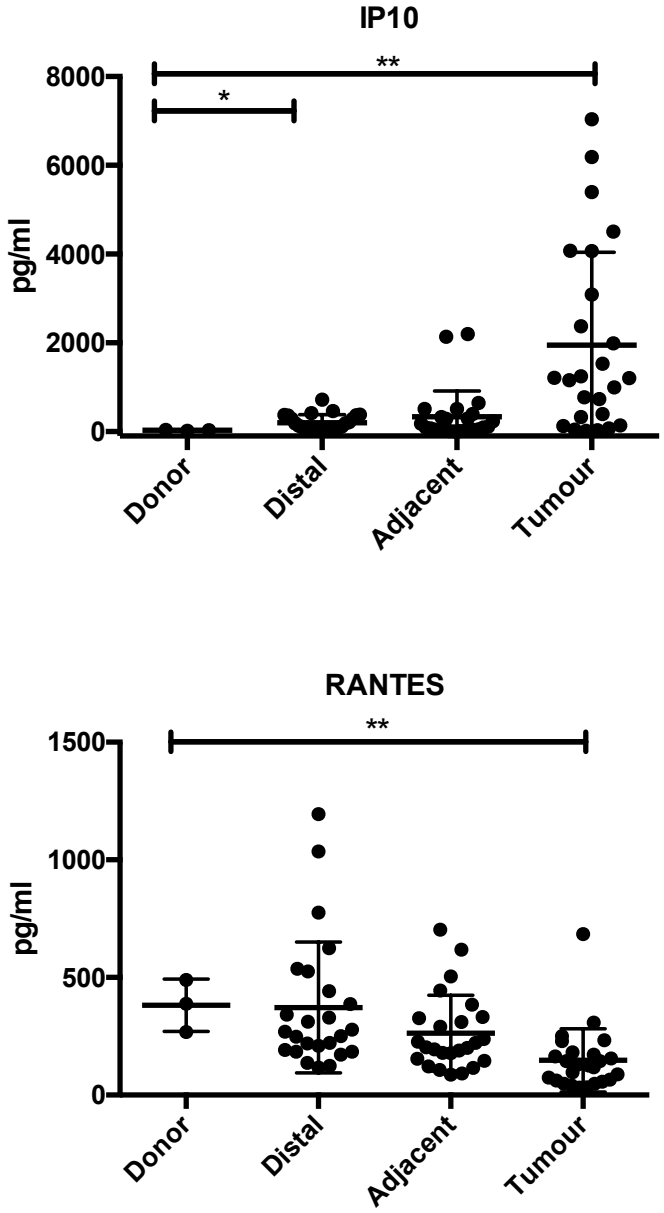
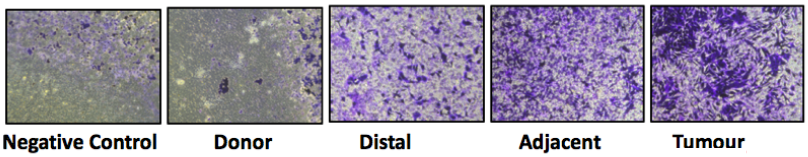


Figure 3.19 Levels of IP10 and RANTES in donor, distal metastatic liver, adjacent metastatic and tumour conditioned media. Cytokines were quantified by Multiplex ELISA in order to establish levels of expression across metastatic liver. Results were analysed using ANOVA with Tukey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001.

3.3.5 The cytokine microenvironment of metastatic liver promotes migration of metastatic CRC cells

To define functional effects of the altered cytokine profile of metastatic liver, a Boyden chamber migration assay was performed. Metastatic CRC cells (SW620) were seeded at a density of 2.5 $x10^4$ cells in the upper compartment of Matrigel-coated inserts, liver-conditioned medium was used as chemoattractant in the lower chamber. After 48h of incubation, cells on the lower face of the chambers were fixed using 1% glutaraldehyde, stained with 0.1% crystal violet, and imaged under a light microscope at a magnification of 10X. SW620 cells migrated towards the CM from all areas of metastatic liver (tumour, tumour adjacent and distal liver) with similar affinity, that was significantly increased compared to donor liver CM (Figure 3.19).



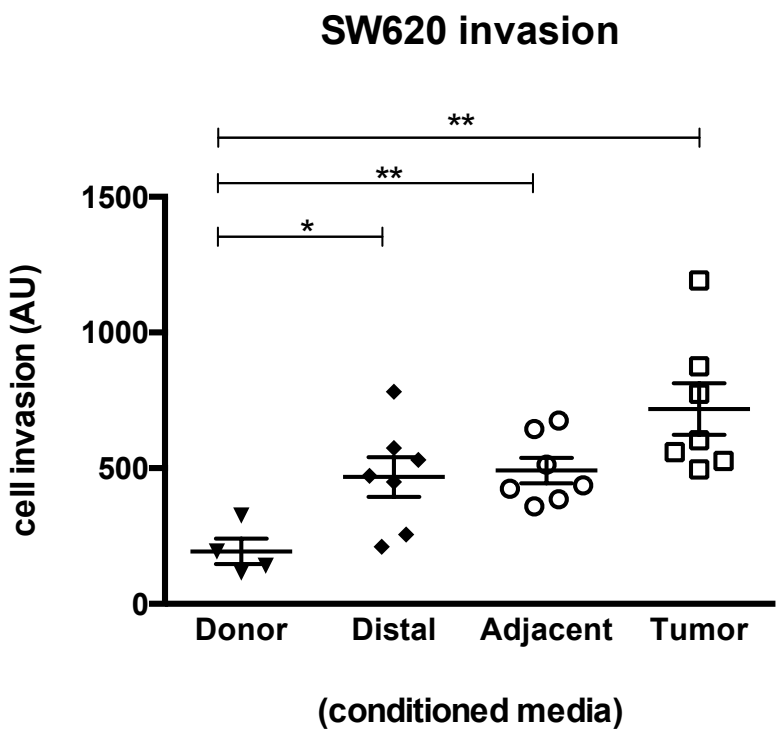


Figure 3.20 Boyden chamber migration assay. Crystal violet staining was quantified using ImageJ software (U.S. National Institute of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/), and cell invasion was expressed as percentage compared to control inserts with no chemoattractant. Results were analysed using ANOVA with Turkey's multiple comparisons test, *p<0.05; **p <0.01 ***p <0.001

5.3 Discussion

The tissue microenvironment of metastatic liver is characterised by increased levels of inflammatory cytokines and perturbation of chemokine expression. This increased local inflammation may disrupt the normal hepatic immune cell repertoire and subsequent tumour surveillance. We have found depletion of PMN in the hepatic parenchyma to be predictive of intrahepatic disease recurrence. Furthermore, CM from all areas of metastatic liver are highly chemotactic for metastatic colon cancer cells (SW620). Local hepatic cytokine and growth factor interactions are critical to the phenotype and function of tissue resident immune cells (Weiskirchen R, 2014; Harmon C, 2016). Ultimately, it is the altered cytokine landscape that drives changes in the immune repertoire, promoting malignant cell migration and ensuing disease recurrence.

Healthy human liver is a dynamic organ, undergoing constant inflammation in the maintenance of homeostasis (Robinson MW, 2016). Here we demonstrate a broad range of inflammatory and immune regulatory cytokines and chemokines present in non-pathologic donor liver. Elevated levels of chemokines such as CCL5, CCL2 and IL-8 facilitate leukocyte accumulation (dos Santos AC, 2005; Vilela MC, 2009; Muthuswamy R, 2012; Turner MD, 2014) and correlate to the increased levels of innate T cells, NK cells and monocytes seen in healthy liver (Dupaul-Chicoine J, 2015; Brackett CM, 2016). Furthermore, elevated levels of resistin, lipocalin, MIF and DPP4 were observed, confirming continual active tissue remodelling in healthy human liver (White DA, 2014; Wagner L, 2016; Moschen AR, 2017). Successful establishment of metastatic disease relies on manipulation of this dynamic immune microenvironment.

Cytokines and chemokines are increasingly recognized as integral to malignant progression, not only for their ability to induce tumour cell migration but also in their ability to shape an inflammatory niche that is tumour protective. Our analysis shows

increasing concentrations of CXCL10 with proximity to tumour deposits. A known chemoattractant for T cells, these data echo recent findings of increased T cells at the invasive margin of CRLM, orchestrated by CXCL10 (Halama N, 2016). Further analysis of the hepatic parenchyma of metastatic liver revealed increased levels of CXCL5, IL-6, CXCL1, G-CSF, GM-CSF and CCL3 when compared with healthy donor liver. These factors, involved in neutrophil differentiation (Furze RC, 2008), migration (Ahuja N, 2012; Asfaha S, 2013) and activation (Mantovani A, 2011) provide evidence that in the presence of malignancy, the whole hepatic immune landscape undergoes remodelling (Lee YS, 2012; Wang D, 2017). Density and type of tumoural immune cell infiltrate have demonstrated prognostic significance in CRC (Galon J, 2006), and its metastases (Pugh SA, 2014; Berthel A, 2017). Indeed, our examination of histologically uninvolved hepatic parenchyma of CRLM revealed significant alterations in immune cell ratios with an elevated lymphocyte and decreased granulocyte count relative to donor healthy liver (Figure 3.9). This may be due to changes in the chemokine landscape, characterized by increased expression of CCL5 in distal tissue.

PMN play a key role in host defence by mobilizing to a site of insult to phagocytose invading organisms. In inflamed tissues neutrophils have been shown to engage in bi-directional interactions with macrophages, lymphocytes and mesenchymal stem cells, affecting cell proliferation, differentiation and survival (Jaillon S, 2013). Furthermore, peritumoral PMN are associated with improved prognosis and increased sensitivity to 5-Fluorouracil based chemotherapy in the setting of CRC (Galdiero MR, 2016). Conversely following surgical stress, neutrophil extracellular traps have been implicated in the formation of liver metastases (Tohme S, 2016). The role of PMN in the context of malignancy remains complex, and may confer a pro- or anti-tumourigenic effect (Treffers LW, 2016). Much like macrophages, Fridlender et al. have described plasticity in tumour-

associated neutrophils whereupon a N1 or N2 phenotype might prevail depending on host conditions (Fridlender ZG, 2012). Following cytokine activation, tumour cytotoxic N1 neutrophils have the potential to kill malignant cells thus inhibiting further tumour growth (Mishalian I, 2014; Shaul ME, 2016). Examination of our cohort demonstrated depleted neutrophil densities throughout metastatic liver. Moreover, hepatic metastatic recurrence was seen in those with significantly lower neutrophil densities (Figure 3.9, Figure 3.10) implicating a protective role for hepatic neutrophils in the presence of metastatic disease.

We have found the cytokine milieu present in all areas of metastatic liver to be chemotactic for metastatic CRC cells *in vitro* (Figure 3.20). Moreover, the most prevalent cytokines found exhibit a predilection for granulocyte migration and function. Overexpression of granulocyte-related cytokines across metastatic liver is reflected by changes in hepatic immune cell composition, with recurrent intrahepatic disease seen in those either unable to mobilise PMN to the hepatic parenchyma, or in whom granulocyte trafficking to metastatic deposits rendered the parenchyma depleted of PMN. Analysis of peritumoral immune infiltrate has been previously proven as an accurate prognostic predictor in CRC (Galon J, 2014) such that routine measurement of tumoural immune infiltrates is recommended as an adjunct to current staging systems (Kirilovsky A, 2016). Immunoscores in metastatic colon tumours have shown consistent correlation to overall survival regardless of tumoural KRAS status (Kwak Y, 2016). Similarly, components of the systemic inflammatory response reflect tumour progression and metastasis (Proctor MJ, 2010). Elevated neutrophil lymphocyte ratios, associated with a specific pro-inflammatory cytokine signature in plasma, correlate with poor disease specific and overall survivals in both primary CRC (Dolan RD, 2018), and CRLM (Tang H, 2016). Altered immune cell ratios influence the activity of tumour surveillance mechanisms

present in metastatic liver, the effects of which extend beyond the tissue planes resected at “curative” hepatectomy. Targeting the complex immune interactions in the hepatic parenchyma in this clinical setting, may provide new therapeutic options for recurrent metastatic disease.

Chapter 4: Discussion

Metastatic colorectal cancer remains a compelling health problem, despite significant strides in treatment modalities over the last two decades. This analysis of patients undergoing hepatectomy for CRLM has shown the importance of the host immune response to malignancy in shaping patient outcomes. Examination of patient's preoperative blood revealed a negative association between elevated neutrophil counts and neutrophil lymphocyte ratio and subsequent survival. The inverse of which was seen on histological analysis of metastatic hepatic parenchyma whereby depleted neutrophil counts were associated with shorter time to disease recurrence.

Owing to its anatomical location, the liver is primed as the most common site of metastatic development. While local radiation and ablation therapies may temporise the disease process, surgical resection remains the only chance to cure. In recent years, systemic chemotherapy has increasingly been used as part of a multidisciplinary strategy to select out a more eligible subgroup of patients for surgical resection. This has led to increasing numbers of patients undergoing hepatic resection for CRLM. In spite of this, almost two thirds of patients present with recurrent metastatic disease following resection. In essence, the contemporary treatment paradigm is slowing but not eradicating disease progression. CRLM remain a highly heterogenous spectrum of tumours with wide variability in response rates to the current therapies available. Despite various scoring systems to stratify prognosis, there remains a paucity of accurate biomarkers to predict the clinical course of these patients. The host immune response to malignancy is emerging as a critical factor in the progression of metastasis. In this thesis we explored the immune profile of patients with CRLM to establish if specific immune signatures correlated to shorter time to disease recurrence or death.

For metastasis to occur, the primary tumour expands different myeloid cell populations in the liver, "priming" the destination organ to create a pre-metastatic niche. In a murine

model of pre-invasive abdominal tumours, myeloid cells are recruited to the liver by chemokine expression from the tumour. In turn these hepatic myeloid cells inhibit cytotoxic T lymphocytes, potentiating the pro-metastatic niche (Connolly MK, 2010). Colorectal tumours have demonstrated this by recruiting CCR1+ myeloid cells through CCL15, or CCR2+ myeloid cells through CCL2 to enable metastatic development in the liver (Itatani Y1, 2013, Zhao L, 2013). Healthy liver however has a robust and complex immune system with lymphoid and myeloid populations playing key roles in maintaining a healthy microenvironment and providing protection against malignancy. As damage associated molecular patterns (DAMP) and microbial associated molecular patterns (MAMP) arrive at the liver, Kupffer cells (via pattern recognition receptors) bind them stimulating phagocytosis without producing an inflammatory response (Dixon LJ, 2013). Contributing significantly to the healthy local microenvironment, Kupffer cells also play a key role in hepatic regeneration and tissue remodelling as well as immune regulation. Dendritic cells found in healthy liver have been shown to stimulate T cell responses as well as produce an abundance of anti-inflammatory cytokines (Sozzani S, 2017)). While classic neutrophils are found in the liver in response to ischemia, infection and inflammation, myeloid-derived suppressor cells (MDSC) who have a number of common cell surface markers with granulocytic neutrophils, are found in healthy liver. These cells have the ability to block T cell expansion but given their shared cell surface markers and similar morphology to granulocytic neutrophils, a number of groups have questioned whether they are two distinct entities, or a single cell displaying plasticity (Zhou J, 2018; Tcyganov E, 2018).

Though not commonly encountered in healthy liver, neutrophil-like granulocyte populations are key players in the hepatic tumour microenvironment (Zhou SL, 2019). Substantially increased numbers of circulating neutrophils are classically found in the

blood of patients with advanced malignancy and are associated with poor disease specific outcomes (Templeton AJ, 2014). In our analysis of patients undergoing hepatectomy for CRLM, we demonstrated an association between preoperative blood neutrophil and lymphocyte counts and postoperative survival. These results confirm the findings of other published studies (Giakoustidis A, 2015) (Neal CP, 2015) whereby a high neutrophil to lymphocyte ratio (NLR) was predictive of poor outcome following resection of CRLM. Whilst there is a correlation between high NLR and increased systemic inflammatory activity including high circulating levels of IL8, PDGF and G-CSF, the mechanisms that underpin the association between malignancy and elevated NLR are poorly understood. Increased circulating neutrophils have been shown to impede the cytotoxic activity of NK cells and lymphocytes thus impairing the antitumour response (Bassani B, 2019). Additionally, elevated NLR is associated with T cell-derived IL17 which indirectly stimulates MDSCs to suppress T cell proliferation (Motomura T, 2013).

Upon further analysis, in patients who received neoadjuvant chemotherapy, we found that NLR was not predictive of outcome. A similar phenomenon has previously been described in breast cancer patients whereby NLR was not predictive of outcome in those who underwent neoadjuvant chemotherapy (Suppan C, 2015). Neoadjuvant chemotherapy induces increased numbers of TILs in the tumour microenvironment which may significantly change anti-tumour activity (Pelekanou V, 2017). The local hepatic immune profile is similarly influenced, with increased numbers of CD3+ lymphocytes found in liver metastases following treatment with neoadjuvant chemotherapy (Tanis E, 2015). We also found that elevated NLR in patients undergoing repeat resection for recurrent metastases failed to correlate with any marker of prognostic value. Surgery diminishes cell mediated immunity with reduced lymphocyte proliferation

along with suppression of TNFα, IFNγ and IL2 in the immediate postoperative phase (Amodeo G, 2018). However, the immune composition of the post-operative liver remains elusive as does the time to recovery of the healthy hepatic immune microenvironment. Indeed, whether the hepatic immune microenvironment re-establishes following surgical insult remains to be proven.

With this in mind, the next part of our study aimed to characterise the immune composition of the liver parenchyma in patients with CRLM. An altered cytokine profile was demonstrated in metastatic liver when compared to control liver on protein array. Specifically, 12 cytokines quantified by multiplex ELISA demonstrated differential expression across metastatic liver at distances from the tumour deposit. Specifically increased concentrations of LIF, GMCSF and IP10, potent stimulators of granulocyte migration and function were found in the metastatic microenvironment. This reinforces the concept that the immune landscape of the liver is manipulated in the presence of metastases. However, the source of these cytokines has yet to be determined. Whether the tumour itself secretes cytokines to attract granulocytes to its microenvironment and potentiate its pro-metastatic niche, or, whether the tumour microenvironment influences hepatic resident immune cells to produce cytokines and chemokines directing migration of granulocytes remains unknown.

The overexpression of granulocyte-related cytokines observed in metastatic liver was accompanied by changes in hepatic immune cell composition. Histological quantification demonstrated increased numbers of lymphocytes and decreased numbers of neutrophils in metastatic liver parenchyma when compared to donor. On follow up, 52% of our patient cohort developed recurrent hepatic metastases. Recurrent disease

correlated closely with depleted neutrophils from the hepatic parenchyma. In addition, shorter time to metastatic recurrence was demonstrated in these patients. With this finding, and with respect to significantly increased neutrophil densities found in donor liver when compared to metastatic parenchyma, we propose a protective role conferred by this hepatic neutrophil population. Studies on the role of neutrophils within the tumour microenvironment remain ambiguous. Able to migrate from the systemic circulation into tissues under the influence of specific cytokines and chemokines, high levels of intratumoural neutrophils are associated with shorter disease free and overall survival for certain malignancies (Rakaee M, 2016). Nonetheless, under certain conditions, neutrophils exhibit direct antitumour effects. Fridlender et al describe two clearly distinct neutrophil phenotypes that are either pro-tumourigenic (N2) or antitumourigenic (N1) depending on the local microenvironment (Shaul ME, 2018) Furthermore, a potently cytotoxic phenotype was demonstrates in neutrophils associated with early tumours where much weaker cytotoxic effects were exerted by neutrophils associated with established tumours. It is possible that metastatic recurrence occurs in those either unable to mobilise neutrophils to the hepatic parenchyma, or in whom granulocyte trafficking to metastatic deposits rendered the parenchyma depleted of neutrophils. While we found neutrophil depletion in metastatic liver negatively correlated with patient survival, this was a histological finding and thus no functional assays were performed. To this end, analysis of metastatic liver to isolate and establish the function of these neutrophils would further delineate their contribution to ongoing disease in this setting.

Understanding the complex interactions that occur between the immune cells and inflammatory mediators in metastatic liver has many potential benefits for treating this

disease. Firstly, molecular classification of tumours according to their immune profile will allow more accurate prognostication of these patients, and enable judicious use of existing chemotherapy regimens. Secondly, understanding the key mediators of immune suppression in the microenvironment of metastatic liver will highlight potential biomarkers and enable the development of targeted and more effective therapies. Cancer immunotherapy is undergoing a resurgence with the FDA recently approving a slew of immune based products. Ipilimumab an antagonist antibody against cytotoxic T-lymphocyte antigen-4 (CTLA-4) has been licenced for treatment of advanced melanoma (Hodi FS, 2016), furthermore pembrolizumab has been licenced for use in refractory or metastatic MSI-H CRC (Marginean EC, 2018). Despite these advances, the ability to initiate therapeutically relevant antitumour immune responses in cancer patients is challenging. Tumour cells employ a variety of immune evasion mechanisms, which represent a fundamental barrier to the success of cancer immunotherapy. An accurate understanding of the tumour microenvironment and its suppressive properties will provide us with the tools to overcome these mechanisms or more effectively tailor therapies to an individual. In order to better stratify tumours and predict response to therapy, there is an international consensus that immune scores should be incorporated into the TNM cancer classification system (Kirilovsky A, 2016). However, the inherent complexity of quantitative immunohistochemistry, in conjunction with variable assay protocols across laboratories, the many immune cell types analysed, regional selection criteria and variable immune infiltration quantification methods underscore the urgent need to reach assay harmonization. An alternative is to identify another assay that will accurately capture the immune status of a patient.

To conclude, this work identified alterations in immune cell microenvironment in the presence of metastatic disease of the liver . The effects of the metastatic microenvironment are evident across the liver as seen in differing immune cell frequencies and alterations in cytokine concentrations within the organ. As a result of these alterations, metastatic liver is chemo attractive to metastatic CRC cells. Recurrent colorectal liver metastases remain common and are associated decreased neutrophil frequencies in metastatic hepatic parenchyma. In order to abrogate recurrent metastatic disease, it is critical to understand and manipulate the underlying immune cell changes present in metastatic liver. This present body of work highlights the importance of neutrophils within the hepatic parenchyma as a possible therapeutic target.

Future Projects

The findings of this work have provided a platform for a number of further studies.

1. Primary colorectal tumours are thought to expand different myeloid cell populations in the liver, "priming" it for the development of metastases. Analysis of the cytokine profile of healthy liver and serum in patients with early stage colorectal cancer may identify the key cytokines and chemokines in the "priming" process.

2. Functional and phenotypic characterisation of granulocyte-like immune populations in healthy liver

3. A number of cytokines including GMCSF, LIF, and IL6 were found to be differentially expressed in metastatic liver when compared to donor. Determining the source and effect of these cytokines on hepatic immune cells, may identify new biomarkers.

4. Additionally, further functional work where specific cytokines which we identified in metastatic liver are either suppressed or stimulated may clarify their function within the tumour microenvironment.

5. Depleted hepatic parenchymal neutrophils were associated with metastatic recurrence and shorter time to recurrence. Isolation of these cells with functional studies may help further clarify their role in the metastatic process.

6. Replication of metastatic liver disease in an animal model with administration of exogenous neutrophils may establish a therapeutic role in this setting.

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