Exploring Circulating Biomarkers in Patients with Hepatocellular Cancer

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Abstract

Hepatocellular cancer (HCC) is the sixth commonest cancer worldwide and the second leading cause of cancer mortality. The majority of patients present with advanced disease, where cure is not an option and palliative therapies are limited. What is more, biomarkers to stratify available therapies effectively are lacking. Tissue is not routinely obtained as diagnosis of HCC is largely reliant on imaging, as this is sensitive and specific in the majority of patients with cirrhosis and liver biopsy carries small but recognised risks - including tumour seeding and bleeding. Serum AFP lacks HCC diagnostic sensitivity and specific, but remains the only clinically useful serum biomarker – employed despite its limitations in surveillance programmes as well as to monitor HCC progression and to stratify patients for therapy. This highlights the need for alternative biomarkers for HCC management. Sampling patient blood is a quick, non-invasive and inexpensive method and may be regarded as a 'liquid biopsy' if it could deliver clinically relevant molecular information about the tumour or its microenvironment. Several liquid biopsy methods have been explored: circulating tumour cell (CTC) detection and characterisation; circulating tumour DNA (ctDNA) KRAS mutation detection; circulating immune cell counts and gene expression signatures of peripheral blood mononucleocytes (PBMCs).

CTCs can be detected in patients with cancer and have the potential to provide diagnostic, prognostic and treatment stratifying information. A method for CTC detection using the Imagestream – an imaging fluorescent activated cells sorter with multi-channel immunofluorescence - was developed and optimised. A CD45-immunomagnetic depletion resulted in ≥95% reduction in WBCs with the maintenance of a recovery rate of 51.3-65.37% of CTCs. The remaining cell suspension was labelled with a panel of fluorescent antibodies to enable cell characterisation prior to running through the Imagestream.

Between 1 and 1642 CTCs were detected in 65% of HCC patients. The presence of CTCs indicated a worse median overall survival (OS) in HCC patients (24.5 months vs 12 months, Log-Rank p>0.0001). Expression of biomarkers on CTCs was heterogeneous with CK being the most commonly detected biomarker, followed by DNA-

PK. In some patients, CTCs observed were negative for all of the biomarkers in the panel but detectable on the basis of size and morphology. Clusters of CTCs and leucocyte interactions with CTCs were also observed.

Studies on plasma ctDNA detected KRAS mutation in 2/38 (5%) of patients with HCC.

Retrospective patient blood data analysis demonstrated that circulating neutrophils were the key circulating immune cell driving HCC progression. In two patient cohorts – Newcastle (n=583) and Hong Kong (n=585), circulating neutrophil counts were an independent predictor of prognosis. Furthermore, in the combined cohort (n=1168), the neutrophil count, either alone or combined with platelets and lymphocytes, was associated with significant differences in patients survival.

In a small pilot cohort, gene expression analysis of PBMCs identified NFAT in the immune response as being the top altered pathway in patients with NAFLD/NASH-HCC. DNA extracts prepared from PBMCs in patients with NAFLD-NASH-HCC were more similar to samples derived from patients with HCV-HCC compared to non-cancerous NAFLD/NASH controls.

These pilot data were encouraging, supporting a potential role for future application of liquid biopsy tools in the clinical setting for patients with HCC.

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Abbreviations

AA	Acetaldehyde
AASLD	American Association for the Study of Liver Diseases
AFP	Alphafetoprotein
AIH	Auto-immune hepatitis
Alb	Albumin
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
ALIOS	American Lifestyle-Induced Obesity Syndrome
ALL	Acute lymphoblastic leukaemia
ARG1	Arginase 1
Array-CGH	Array-comparitive genomic hybridisation
AST	Aspartate aminotransferase
BCLC	Barcelona Clinic liver cancer
BEAMing	Beads, emulsions, amplification, and magnetic technology, sensitive mutation detection
bFGF	Basic fibroblast growth factor
Bili	Bilirubin
b-raf	b-rapidly accelerated fibrosarcoma
Breg	B regulatory
BSA	Bovine serum albumin
CA19-9	Cancer antigen 19-9
CAH	Chronic active hepatitis
CCA	Cholangiocarcinomas
CCD	Charge coupled device
CCL	Chemokine C-C motif ligand
CD45	Common leucocyte antigen
cDNA	Complementary DNA
CECs	Circulating endothelial cells
cfDNA	Circulating free deoxyribonucleic acid
cfNA	Circulating free nucleic acid
cfRNA	Circulating free ribonucleic acid

CGH	Comparative genomic hybridisation
CK	Cytokeratin
CLD	Chronic Liver Disease
CLIP	Cancer of the liver Italian programme
CLL	Chronic lymphocytic leukaemia
COX 1/2	Cyclooxygenase
CRCa	Colorectal cancer
CT	Computed tomography
CTC	Circulating tumour cell
CTM	Circulating tumour microemboli
CUPI	Chinese University prognostic index
CXCL	Chemokine (C-X-C motif) ligand
DAA	Directly acting antiviral agents
DAPI	4',6-diamidino-2-phenylindole
DEB	Drug-eluting bead
DEN	N-nitrosodiethylamine
DEP	Dielectrophoresis
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSBs	Double strand breaks
DTC	Disseminated tumour cell
EASL	European Association for the Study of Liver
ECM	Extracellular matrix
ECOG	Eastern cooperative oncology group
EDF	Extended depth of field
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EORTC	European organisation for research and treatment of cancer
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-related kinases
FACS	Fluorescence-activated cell sorting
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor

FISH	Fluorescent *in situ* hybridisation
FNA	Fine needle aspiration
FoxP3	Forkhead box P3
GC33	Glypican-3 humanised monoclonal antibody
GPC3	Glypican-3
GRETCH	Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire
HB-chip	Herringbone chip
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepPar1	Hepatocyte Paraffin 1
HER-2	Human epidermal growth factor-2
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1α
HR	Hazard ratio
HRAS	Harvey rat sarcoma viral oncogene homolog
HSPG	Heparan sulphate proteoglycan
hTERT	Human telomerase reverse transcriptase
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-22	Interleukin-22
IL-6	interleukin-6
iNOS	Inducible nitric oxide synthase
INR	International normalised ratio
ISET	Isolation by size of epithelial tumour cell
IU	International units
JIS	Japan integrated staging
kDa	Kilo Daltons
KRAS	Kirsten rat sarcoma viral oncogene homolog
LED	Light emitting diode
LSECs	Liver sinusoidal endothelial cells
M1	Macrophages pro-inflammatory
M2	Macrophages immunoregulatory

MAPK	Mitogen activated protein kinase
MEK1/2	Mitogen-activated protein kinase kinase (alias MAP2K, MAPKK)
MELD	Model for end-stage liver disease
MEMs	Micro- electro-mechanical system
MET	Mesenchymal-to-epithelial transition
MetS-HCC	Metabolic syndrome-associated HCC
MMPs	matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTOR	Mechanistic target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor κB
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NK	Natural killer
NKT cells	natural killer T-cells
NLR	neutrophils to lymphocytes
NOD/SCID	Non-obese diabetic/ severe combined immunodeficient
NOPE	Neighbour of punc-E11
NRAS	Neuroblastoma rat sarcoma viral oncogene homolog
NSAIDs	Non-steroidal anti-inflammatories
OS	Overall survival
PBC	Primary biliary cirrhosis
PBMCs	Peripheral blood mononucleocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Pegylated interferon
PEI	Percutaneous ethanol injection
pERK	Note ERK already in list
PFS	Progression-free survival
PI3K	Phosphatinodylinositol 3-kinase
PLR	Platelet to lymphocyte ratio

PSA	Prostate-specific antigen
PST	Performance status
PT	Prothrombin time
PVT	Portal vein thrombosis
qPCR	Quantitative polymerase chain reaction
RAS	Rat sarcoma
RCT	Randomised controlled trial
RFA	Radiofrequency ablation
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RT	Room temperature
RTKs	Receptor tyrosine kinases
SHARP	Sorafenib hepatocellular carcinoma assessment randomised protocol
SH-HCC	Steatohepatitic–hepatocellular cancer
shRNA	Short hairpin RNA
SII	Systemic immune-inflammation index
SIRT	Selective internal radiation therapy
SNPs	Single nucleotide polymorphisms
Spred	sprout-related protein with Ena/vasodilator-stimulated phosphoprotein homology-1 domain
STAT3	Signal transducer and activator of transcription 3
SULF1	Sulfatase 1
SULF2	Sulfatase 2
T2DM	Type 2 diabetes mellitus
TACE	Trans-arterial chemo-embolisation
TACSTD1	EpCAM (alias)
TAE	Trans-arterial embolisation
TAFs	tumour-associated fibroblasts
TAMs	Tumour-associated macrophages
TANs	Tumour associated neutrophils
TERT	Telomerase reverse transcriptase
TGFβ	Transforming growth factor β
Th	T-helper
TILs	Tumour infiltrating lymphocytes

TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
TNFα	Tumour necrosis factor α
TNM	Tumour, node, metastasis
Treg	T regulatory
TTP	Time to progression
USS	Ultrasound scan
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor
WBCs	White blood cells
WCC	White cell count
ZEB1	Zinc finger E-box binding homeobox 1

Chapter 1. Literature review

1.1 Introduction to HCC

Primary liver cancer constitutes of: cholangiocarcinomas (CCA) that originate from the bile duct epithelium; hepatocellular carcinomas (HCC) that originate from hepatocytes; hemangiosarcomas that arise from mesenchymal tissues, as well as hepatoblastomas, bile duct cystadenocarcinomas and epitheliod haemangioendotheliomas (Farazi and DePinho, 2006). Of all primary liver cancers, HCC is the most common primary liver cancer accounting for approximately 80-90% of all cases (El-Serag and Rudolph, 2007, Farazi and DePinho, 2006).

1.1.1 Epidemiology of HCC

HCC is the sixth most frequent cancer worldwide and the second most common cause of cancer mortality (Jemal et al., 2011). It has a higher prevalence in males compared to females with a male to female ratio of 2.4:1 (Di Bisceglie, 2009, Ferlay et al., 2010). In the United Kingdom, liver cancer is the 18th most common cancer and rates have increased by 60-70% from 2003 to 2012 (Office for National Statistics, 2012). Incidence increases with age; in the UK the average age of onset is 66 years old (Ryder, 2003). The overall incidence of HCC is rising, although its global prevalence geographically is variable with higher rates observed in the developing world such as Eastern and South Eastern Asia as well as Middle and Western Africa compared to developed regions (Ferlay et al., 2010, Jemal et al., 2011). Since the introduction of the hepatitis B virus (HBV) vaccination programmes in the 1980's (Beasley et al., 1983, Lo et al., 1985) and the implementation of new treatments leading to eradication of hepatitis C virus (HCV) (Lange and Sarrazin, 2015) it is likely that the burden of viral-induced HCC will decrease; on the other hand, an increase in obesity and associated metabolic risk factors is likely to lead to an increase in HCC with a non-alcoholic fatty liver disease (NAFLD)/ non-alcoholic steatohepatitis (NASH) aetiology (Starley et al., 2010, Dyson et al., 2014).

1.1.2 Aetiology of HCC

In 80% of cases, HCC develops on a background of cirrhosis or advanced liver fibrosis (Bosch et al., 2004, Severi et al., 2010, van Malenstein et al., 2011). It is well established that infection with HBV and HCV are contributing factors to the development of HCC (Severi et al., 2010). The carcinogenic effect of prolonged excess alcohol consumption leading to alcoholic liver disease (ALD) increases the risk of HCC development (Morgan et al., 2004, Stickel et al., 2002). The development of cirrhosis is a concern in patients with NAFLD and NASH (Severi et al., 2010). Congenital disorders such as haemachromatosis and Wilson's disease also contribute to cirrhosis or advanced fibrosis (Severi et al., 2010). Of the remaining cases of HCC that arise independently of cirrhosis or advanced fibrosis, risk factors include aflatoxin B1, exposure from contaminated food sources and the use of anabolic steroids or oral contraceptives (Bosch et al., 2004, Severi et al., 2010).

1.1.3 Hepatitis virus-induced HCC

Infection with either HBV/HCV are major causes of chronic liver disease associated with the development of 80% of cases of HCC (El-Serag, 2012). Viral hepatitis B and C induced cirrhosis is associated with an increased risk of HCC development compared to other aetiologies (Perz et al., 2006). Viral hepatitis caused by HBV or HCV infection leads to an inflammatory immune response in the liver leading to fibrosis and cirrhosis. HBV may exert mutagenic effects by integrating into the host genome and causing genomic instability (Blum and Moradpour, 2002).

Chronic HBV infection is estimated to be present in 5% of the world population with prevalence varying between 0.1% and 20% between different geographical regions (El-Serag, 2012, Mohr et al., 2015). Overall, HBV accounts for over 50% of HCC cases worldwide, rising to 70-80% of cases in highly endemic areas (Nguyen et al., 2009). Prophylaxis of HBV has been made possible by the introduction of vaccines against HBV, first introduced in the 1980s (Di Bisceglie, 2009). The success of implemented vaccination programmes has led to a decline in incidence rates (Chang et al., 1997, Ni

et al., 2001). It is predicted that with continuation of HBV vaccination programmes, the number of HCC cases attributed to HBV will continue to decrease.

HCV is another leading cause of HCC development. Akin to HBV, the incidence of HCV has varying prevalence globally. HCV is the major risk factor for HCC patients in Japan where it accounts for approximately 80% of cases of HCC (Yoshizawa, 2002). Advances in directly acting antiviral agents (DAAs), in combination with pegylated interferon (PEG) α and ribavirin are likely to lead to the eradication of HCV in a large proportion patients (Lange et al., 2014).

1.1.4 Alcoholic liver disease

Prolonged excess alcohol intake has been reported to increase the risk of HCC ~5-fold (Morgan et al., 2004). The risk of HCC increases proportionally with the increased level of alcohol intake (Donato et al., 2002). Alcohol has also been shown to have a synergistic effect with HBV and HCV, leading to increased virus replication (El-Serag and Rudolph, 2007, Stickel et al., 2002). In the UK, alcohol is a major cause of chronic liver disease (CLD), resulting in liver injury and subsequent cirrhosis (Dyson et al., 2014). The effects of alcohol in the development of ALD are not entirely understood but alcohol can exert its effects at the DNA level (Morgan et al., 2004, Stickel et al., 2002). In the liver, alcohol is first metabolised to acetaldehyde (AA) by hepatic enzymes alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1) (Stickel et al., 2002). AA adducts bind readily to cellular proteins and potentially to DNA exerting a mutagenic and carcinogenic effect (Stickel et al., 2002). Alcohol metabolism also leads to the production of free radicals which may be DNA-damaging or result in alterations in DNA methylation and expression (Morgan et al., 2004, Stickel et al., 2002). The toxicity of alcohol and ethanol metabolites can also lead to the activation of inflammatory cells through the increase in reactive oxygen species (ROS) and stimulation of inflammatory cytokines (Niederau, 2015). In addition to the carcinogenic effects of alcohol, malnutrition and vitamin deficiencies associated with alcoholism may also be contributory to an altered immune response (Stickel et al., 2002).

1.1.5 NAFLD and NASH

NAFLD is the clinical manifestation of metabolic syndrome in the liver. Features of metabolic syndrome include: impaired fasting glucose, type 2 diabetes mellitus (T2DM), dyslipidaemia, central obesity, and hypertension. In the liver, NAFLD is histologically defined as excess steatosis (fat accumulation in greater than 5% of hepatocytes), NASH is a progression of simple steatosis, including inflammation and associated injured or 'ballooned' hepatocytes (steatohepatitis) (Ratziu et al., 2010). NAFLD is now the most predominant cause of CLD in developed countries and a predicted increase in obesity suggests that this trend is going to increase in the upcoming years (Dyson et al., 2014, Starley et al., 2010). It is estimated that over the course of 5-7 years, 3.6-12% of patients with NASH/cryptogenic cirrhosis will develop cirrhosis-related complications including HCC (Starley et al., 2010).

1.1.6 Pathogenesis of HCC

Models of hepatocarcinogenesis have been used in attempt to describe the transition of hepatocytes to pre-neoplastic lesions that develop into HCC (Severi et al., 2010, Thorgeirsson and Grisham, 2002). Although the model of hepatocarcinogenesis differs depending on the nature of injury, between the models there are shared cellular responses including inflammation and oxidative stress mechanisms, as well as common genetic and epigenetic events (Farazi and DePinho, 2006, Pons et al., 2005).

Hepatic fibrosis is the wound healing response of the liver to chronic injury (Hui and Friedman, 2003). Prolonged liver injury reduces the regenerative potential of the liver leading to an accumulation of extracellular matrix (ECM) which is eventually replaced by scar tissue (Hui and Friedman, 2003). A continual increase in cell turnover in response to liver insult results in the sensitisation of cells to mutagenic agents (Severi et al., 2010). In cirrhotic liver, regeneration occurs in a non-uniform manner leading to the creation of hyperplastic nodules (Farazi and DePinho, 2006, Kojiro, 2004). At this point it is suggested that there is some form of genetic instability, leading to the progression of pre-malignant dysplastic nodules that are cytologically abnormal; differing in size, colour and cell density compared to the cirrhotic liver (Farazi and DePinho, 2006, Kojiro, 2004).

Dysplastic nodules can be classed as low-grade or high grade depending on their size, infiltration of fat, increased nuclear density and vascular involvement (Kojiro, 2004). Early stage HCC can be sub-divided into distinctly nodular (encapsulated, moderately differentiated, hypervascular) and indistinctly nodular (well-differentiated and hypovascular (Kojiro, 2004). As early stage HCC develops into advanced stage HCC there is an increase in size and pathologically it becomes less well-differentiated. It can often take on a 'nodule-in-nodule' appearance whereby areas of well-differentiated tissue exist within areas of poorly differentiated tissue (Kojiro, 2004).

1.1.7 Molecular classification of HCC

HCC is highly heterogeneous both inter-tumourally and intra-tumourally. During the process of hepatocarcinogenesis there is an accumulation of mutations, changes in genomic stability, DNA hypomethylation and hypermethylation of CpG islands (Calvisi et al., 2007, Thorgeirsson and Grisham, 2002). HCCs contain multiple chromosomal losses and gains which may encompass tumour suppressor genes e.g. TP53, Retinoblastoma protein (Thorgeirsson and Grisham, 2002). Aberrations in pathways involved in cellular proliferation, differentiation, cell cycle and growth factor signalling have been associated with the process of hepatocarcinogeneis including: Wnt/β-catenin, PI3K/AKT/MTOR, RAS/MAPK, IGF, HGF/MET, EGFR, VEGF and PDGF (Cornellà et al., 2011, Llovet and Bruix, 2008). β-catenin is the most frequently mutated oncogene in HCC and TP53 is the most frequently mutated tumour suppressor gene (Zucman-Rossi, 2010). Genome wide transcriptome analysis of 60 HCC tumours led to the proposal of six HCC subgroups (G1-G6) that are distinguishable on the basis of genomic stability, methylation and associated biological pathways (Zucman-Rossi, 2010). Telomerase reverse-transcriptase (TERT) promoter mutations have been identified as being the most frequent mutation in HCC, present in 40-59% of HCC patients and this mutation may be present in the early stages of hepatocarcinogenesis (Nault et al., 2013, Roberts and Wheeler, 2015)

1.1.8 Diagnosis of HCC

As part of the EASL-EORTC guidelines a surveillance programme to monitor at-risk patients is suggested in attempt to detect HCC at an earlier stage when it can be managed more effectively (Bruix et al., 2001, Ryder, 2003, EASL-EORTC, 2012). Despite the implementation of surveillance programmes, patients with HCC often present at an advanced stage with symptoms including abdominal pain, fatigue, weight loss, jaundice and ascites. Diagnosis of HCC is usually based on imaging e.g. CT, MRI and in some cases ultrasound-guided liver tissue biopsy. In a recent report it was indicated that liver tissue biopsies are not always essential for the diagnosis of HCC (Sherman and Bruix, 2015). Biopsies can be used to confirm diagnosis of HCC, provide molecular characterisation of the tumour and to assess the underlying liver disease. However, the size of biopsy – usually ~15 mm x 1.2-2 mm compared to the overall tumour is often relatively small (Rockey et al., 2009). Therefore, a biopsy is likely to provide only a small snapshot of the complex molecular heterogeneity of HCC and does not necessarily give information about the driver cell population (Bruix et al., 2014). Furthermore, there are associated risks with liver tissue biopsies, commonly: pain at the site of biopsy and the risk of bleeding or tumour cell seeding (Rockey et al., 2009). Other associated risks include infection and injury to nearby organs although these latter risks are rare. Although the incidence of these risks are small, they can have serious implications for the patient including mortality (Rockey et al., 2009). Despite this, liver tissue biopsies are still implemented to confirm diagnosis in instances where radiology is not diagnostic or to assess the aetiology and extent of underlying liver disease (Sherman and Bruix, 2015).

1.1.9 Staging systems in HCC

Multiple systems for the staging of HCC have been introduced and evolved over time **Table 1.1**. While attempts have been made to try and evaluate and validate existing staging systems in different subsets of patients, there is still not one universally accepted system. This is in part owing to the complexity of the disease, but also due to the differences in aetiology of the HCC geographically (Marrero et al., 2010, Pons et al., 2005). The implementation of different staging systems means that it is difficult to directly

compare information from clinical studies. Although TNM (tumour, node, metastases) staging is widely implemented in the staging of different types of cancer, in HCC its utility is limited because it does not include an assessment of liver function or underlying liver disease which are primary determinants of prognosis and useful in guiding therapy. Assessment of the underlying liver disease can be performed using the Child-Pugh score which takes into account ascites, hepatic encephalopathy, albumin, bilirubin and prothrombin time (PT) **Table 1.2**. Staging systems that have been developed **Table 1.1** have taken into account: tumour burden (tumour size, tumour number); liver function (Child-Pugh classification or its constituents); serum alphafetoprotein (AFP); portal vein invasion; the presence of extra-hepatic metastases or lymph node involvement and performance status.

Staging system	Tumour staging	Liver function	Other features
Okuda	Tumour size, liver involvement<50%	Ascites, albumin, bilirubin	-
CLIP	Number of nodules, tumour extent	Child-Pugh	AFP
BCLC	Tumour size, tumour number, PVT	Child-Pugh, bilirubin, portal hypertension	ECOG
GRETCH	Tumour size, satellite nodules, resection margin, vascular invasion	Bilirubin, alkaline phosphatase	Karnofsky, AFP
CUPI	TNM	Ascites, bilirubin, alkaline phosphatase	AFP
JIS	Japanese TNM	Child-Pugh	-
SLiDE	Japanese TNM	-	DCP
Tokyo	Tumour size, tumour number	Albumin, bilirubin	-
BALAD	-	Albumin, bilirubin	AFP, AFP-L3, DCP
ALCPS	Tumour size, PVT, lung metastases	Child-Pugh, bilirubin, alkaline phosphatase, urea	AFP
ALBI	-	Albumin, bilirubin	-

Table 1.1 Staging systems in HCC include Cancer of the Liver Italian Programme (CLIP); Barcelona Clinic for Liver Cancer (BCLC); Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire (GRETCH); French and Chinese University prognostic index (CUPI); Japanese Integrated Staging (JIS); Stage Liver Cancer DCP (SLiDe); bilirubin, albumin, Lens culinaris agglutinin-reactive alpha-fetoprotein (AFP-L3), alpha-fetoprotein (AFP), and des-gamma-carboxy prothrombin (DCP); bilirubin, albumin, lens culinaris agglutinin-reactive AFP, AFP, des--carboxyprothrombin (BALAD); Advanced Liver Cancer Prognostic Score (ALCPS); Albumin-bilirubin (ALBI) grade (Johnson et al., 2015). Portal vein thrombosis (PVT).

Clinical parameter	**Points**		
	1	**2**	**3**
Ascites	None	Slight	Moderate
Encephalopathy (grade)	None	I-II	III-IV
Bilirubin (µmol/L)	<35	35-50	>50
Albumin (g/L)	>35	28-35	<28
INR	<1.7	1.8-2.3	>2.3
Total number of points	**5-6**	**7-9**	**10-15**

Table 1.2 Child-Pugh assessment of underlying liver disease takes into account ascites, hepatic encephalopathy, raised bilirubin, lowered albumin and prolonged INR. INR=International normalised ratio.

Despite there being multiple staging systems for HCC, the Barcelona Clinic Liver Cancer (BCLC) classification system **Figure 1.1** is most widely accepted clinically as well as in clinical trials for new HCC drugs (Bruix and Sherman, 2011).

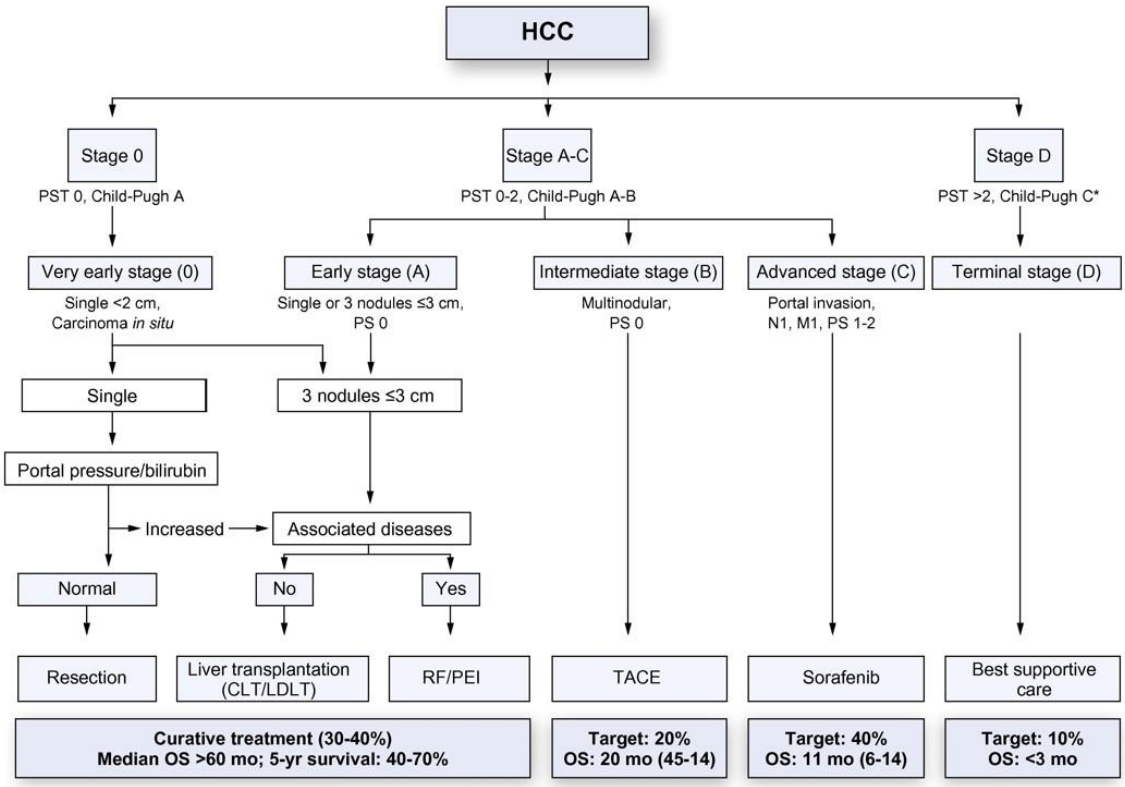


Figure 1.1 BCLC algorithm for the treatment of HCC. From EASL-EORTC Clinical Practice Guidelines (EASL-EORTC, 2012). PS=performance status; CLT=cadaveric liver transplant; LDLT=living donor liver transplantation; PEI=percutaneous ethanol injection; RFA=radiofrequency ablation.

1.1.10 Treatment of HCC

Treatment for HCC is dependent on the stage of the disease, portal pressure and clinical performance (Severi et al., 2010). The BCLC treatment algorithm for HCC is widely clinically adopted and is used in the treatment stratification process **Figure 1.1**. It is considered the gold standard in Europe by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD). Due to HCC often occurring in the background of liver disease, the use of chemotherapeutic agents as treatment is often limited (Farazi and DePinho, 2006).

Treatment can be divided into curative or palliative treatments. Curative treatments include: liver transplant, resection and in some instances, tumour ablation. Although preferred, surgery is not always a suitable option: HCC is often multifocal and technically difficult to resect; transplantation relies on the availability of donors and rates of recurrence post-surgery are high although the latter may in part be due to poor patient selection (Lencioni and Crocetti, 2012, Roayaie et al., 2004). It is estimated that in patients with cirrhosis only around 5% would be suitable for surgery (Lencioni and Crocetti, 2012).

Resection is the recommended treatment option for patients with BCLC stage 0 HCC **Figure 1.1**. Resection leads to removal of the primary tumour but does not resolve the underlying cause of liver disease that is present in the majority of cases and so carries the risk of recurrence. In patients with BCLC stage A HCC, local ablation is considered the next most preferable option in patients that are not suitable for surgery **Figure 1.1**. This is also a potentially curative treatment, the success of which is largely determined by the size of HCC and also its location. Generally patients are considered for this mode of treatment when they have a tumour that is less than 5 cm or there are 3 nodules <3 cm, there is no vascular involvement or metastasis and also have a Child-Pugh score of A or B (Lencioni and Crocetti, 2012). Local ablation therapies include: percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), microwave ablation and cryoablation (EASL-EORTC, 2012). Ablative therapies may be performed percutaneously, laproscopically or as open-surgical procedures and may be carried out under local or general anaesthetic (NHS, 2016). During ablative therapies, imaging technology is implemented to specifically induce cellular damage – by dehydration, protein denaturation or thermal injury – to the tumorous tissue whilst protecting the normal liver (Lencioni et al., 2004). The choice of ablative therapy used is dependent on local guidelines.

In patients where curative treatments cannot be implemented, the palliative treatments of choice include chemoembolization and trans-catheter therapies (EASL-EORTC, 2012). Transarterial chemoembolization (TACE), delivered using drug-eluting beads (DEB) has superseded gel-foam lipiodol particles since its use is associated with less systemic adverse events (EASL-EORTC, 2012). TACE treatment involves the injection of DEBs containing cytotoxic drug – usually cisplatin or doxorubicin – through a catheter

that is inserted through the femoral artery which tracks up to the hepatic artery. Due to the complex nature of the blood supply in the HCC, prior to treatment patients receive a visceral angiogram to identify vessels that require embolization to prevent the delivered treatment spreading to organs such as the lungs and also the peripheral blood supply therefore reducing treatment side effects. Despite this, side effects of TACE are frequent and in cases severe, highlighting the importance to consider patient contraindications prior to treatment (Beaugrand et al., 2004).

Selective internal radiation therapy (SIRT) is an alternative treatment option for patients with non-resectable HCC. This method involves the injection of 32 micron microspheres coated in yttrium - a beta radiation-emitting isotope which causes necrosis of tumour cells usually about 10 days following treatment (Rossi et al., 2010). Trials to compare its efficacy relative to TACE, or in combination with medical therapy, are ongoing.

Few medical treatments have offered survival benefit in patients with HCC despite the recognition of a wide network of cellular signalling pathways involved in the development of HCC **Section 1.1.7**. Currently, Sorafenib (Nexavar, Bayer) – a multi-kinase inhibitor - has been recommended for patients with advanced HCC; showing an improved survival benefit of 2.3-2.8 months in both the SHARP and Asia-Pacific trials (Cheng et al., 2009, Llovet et al., 2008). Sorafenib targets kinases Raf-1 and B-Raf whilst also blocking the activity of the VEGF receptor and PDGF receptor; thus reducing proliferation and angiogenesis. Systemic radiotherapy may also be offered in patients with bone metastases as a palliative treatment to alleviate pain (EASL-EORTC, 2012).

1.1.11 Prognosis

In HCC factors affecting patient survival include, tumour burden, extent of the underlying liver disease, treatment (curative or non-curative) and performance status (Cabibbo et al., 2010, Gallo et al., 1998, Okuda et al., 1985). Currently there is not one single biomarker that is used for the prediction of prognosis in HCC based on the criteria set out by the EASL-EORTC clinical practice guidelines for the management of HCC (EASL-EORTC, 2012). A comparative analysis of seven different staging systems in a US patient cohort was performed to assess the prognostic potential of different staging

systems. The study concluded that although all staging systems showed a decline in survival with increased stage, the BCLC stage had the best predictive potential compared to the other six staging systems (Okuda, TNM, Cancer of the Liver Italian Programme (CLIP), French and Chinese University prognostic index (CUPI), Japanese Integrated Staging (JIS) and Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire (GRETCH) (Marrero et al., 2005). In this patient cohort, consisting mostly of HCC patients with an HCV aetiology and the presence of cirrhosis, the factors impacting survival of non-transplant patients that were independent in a multivariate analysis included: performance status, model for end-stage liver disease (MELD), tumour diameter and portal vein thrombosis (PVT) (Marrero et al., 2005).

1.2 Circulating tumour cells

The concept of circulating tumour cells (CTCs) was first described by Thomas Ashworth in 1869 (Ashworth, 1869). Ashworth microscopically observed cells in the peripheral circulation that resembled those from the primary tumour in a patient with advanced metastatic disease (Ashworth, 1869). CTCs are shed from a primary or metastatic tumour and subsequently circulate in the peripheral bloodstream. In the literature there is also referral to disseminated tumour cells (DTCs), although this description is usually limited to describe tumour cells that reside in the bone marrow (Pantel et al., 2008). A limitation to studying DTCs lies in the acquirement of the sample – bone marrow sampling is not a routine procedure and more invasive in comparison to a blood draw. Clinical implications of the detection and characterisation of CTCs in patients with cancer have been widely implied. However, due to the rarity of CTCs - sometimes as low as ≤1/ml or 1 CTC/1 billion normal blood cells in patients with advanced cancer - it is only with the recent advent of suitable technologies that such investigations are clinically feasible (Allard et al., 2004). Multiple studies have indicated that the detection and characterisation of CTCs may be clinically beneficial by providing novel, less invasive methods of diagnosis; monitoring disease progression; predicting prognosis and guiding and monitoring response to therapy. Further investigation into CTCs may also contribute to understanding the biology of the metastatic process.

1.2.1 CTCs in the process of metastasis

In the majority of patients, the primary tumour is not the principle cause of death – it is more often the case that the patient will die as a result of metastatic disease (Chaffer and Weinberg, 2011). Although the process of metastasis remains to be fully elucidated, it is considered that haematogenous dissemination of CTCs is an early event in the process, occurring following neovascularisation (Parkinson et al., 2012). Two main models have been proposed in attempt to explain the process of metastasis: the linear progression model and the parallel progression model. In the linear progression model the primary tumour is considered the main driver of cancer growth and metastatic cells derive from the primary lesion over a given period of time following accumulation of genetic and epigenetic alterations (Klein, 2009). In contrast, the parallel model assumes

that prior to symptoms or diagnosis of the primary tumour there is early dissemination of tumour cells providing justification for the growth rates of secondary tumours (Klein, 2009).

Not all CTCs/DTCs have the ability to form metastases – it is unknown which characteristics are important and furthermore, how some cells are able to populate at distal sites having evaded detection by the immune system (Klein, 2009). Detection of 'circulating tumour microemboli' (CTM) in blood samples from patients have suggested that CTCs may not always circulate as single cells but may form groups, which are more readily able to form metastatic lesions (Krebs et al., 2010, Vona et al., 2004). It is possible that there is a subset of the CTC population which have a more aggressive phenotype and an increased capability of forming secondary tumours leading to the conclusion that the number of CTCs by itself may not be the best indication of prognosis or response to treatment; instead the molecular characteristics of the CTCs present may be more informative (Sun et al., 2013).

It is difficult to ascertain how representative the CTCs detected are of the primary tumour. It has been noted that there is great intra-tumoural and inter-tumoural heterogeneity and it may be the case that CTCs are neither reflective of the primary tumour or the metastatic lesion. Advances in deep sequencing have now made it possible to identify niche cell populations in a heterogeneous tumour cell population that may have previously gone unnoticed due to amplification of larger cell populations. With the advent of microarray platforms, it is possible to compare the genetic profile of metastatic cells with those derived from primary tumours and it is possible that this can provide information to determine whether or not primary tumours display a metastatic phenotype (Pantel and Brakenhoff, 2004). A study isolated CTCs from patients with colorectal cancer (CRCa) using the CellSearch system and performed array-comparative genomic hybridisation (array-CGH) (Heitzer et al., 2013). In two patients, profiles from the primary tumour, circulating tumour cells and metastases were compared to a panel of 68 CRCa-associated genes resulting in the identification of mutations that were common in the primary tumour and circulating tumour cell population as well as novel mutations that were exclusive to the CTC population (Heitzer et al., 2013).

1.2.2 CTCs and epithelial-mesenchymal-transition

Epithelial-mesenchymal transition (EMT) is an embryonic process that is also thought to play a key role in tumour cell invasion and metastasis by epithelial cells losing their polarity, enabling them to migrate and survive in an extracellular environment (Mima et al., 2013, Thiery, 2002). Characteristically during EMT there is a down-regulation of factors that give the cell its epithelial phenotype such as epithelial cell adhesion molecule (EpCAM), E-cadherin and N-cadherin. Concomitantly there is an up-regulation of mesenchymal markers such as vimentin, Twist, Slug, Snail1/2 and zinc finger E-box binding homeobox 1/2 (ZEB1/2). The reverse process of MET can also occur and it has been hypothesised that in order to metastasise from the primary site, cells may undergo EMT then revert back to an epithelial phenotype at the secondary site. It has been noted that in order for a cell to display a mesenchymal phenotype there are not always dramatic changes in the cell phenotype and importantly, EMT is not a requirement for cells to metastasise (Christiansen and Rajasekaran, 2006). The process of EMT is reliant on embryonic pathways; notably in HCC this includes the wnt/β-catenin pathway and transforming growth factor-β (TGF-β) signalling pathway (Mikulits, 2009).

1.2.3 Clinical utility of CTCs

In a clinical setting there are numerous potential uses of CTCs including aiding in diagnosis, predicting prognosis and guiding and monitoring response to therapy. In the first instance it has been suggested that CTC detection and characterisation in the blood may serve as a 'liquid biopsy' in which characteristics of the tumour are able to be identified instead of using traditional imaging methods or invasive biopsies which carry the risks of tumour spread and bleeding. At this stage the full clinical implications of CTCs remain to be determined, but retrospective studies have shown that CTC enumeration statistically links to clinical parameters such as prediction of progression-free survival (PFS) and overall-survival (OS) in breast, prostate and colorectal cancer (Cohen et al., 2008, Cristofanilli et al., 2004, de Bono et al., 2008). Currently, there is only one US food and drug administration (FDA)-approved CTC detection platform – the CellSearch – which has been clinically validated in metastatic breast, prostate and colorectal cancer (Cohen et al., 2008, Cristofanilli et al., 2004, de Bono et al., 2008). Various other CTC

enrichment methods and detection platforms exist and studies have shown that CTCs may have clinical utility but none of these studies have been verified in large patient cohorts using reliable detection methods that have reached clinical validation.

It has also been proposed that CTCs may have an impact in guiding therapy and measuring response to treatment. While studies have attempted to link the number of CTCs with response to treatment – by taking baseline CTC measurements pre- and post-treatment, there is perhaps a wider application with regards to the characteristics of the CTCs that are observed. Biomarker characterisation of CTCs may be useful in terms of: monitoring treatment response, determining resistance to therapy or in attempt to treat minimal residual disease. The use of biomarkers in the monitoring of treatment is however dependent on the use of a suitable technology that is able to characterise the CTCs with enough confidence to base a treatment decision on. It is difficult to ascertain a suitable threshold when basing this decision on enumeration and it is difficult to assess particular cases which fall on the threshold, especially if this would result in withholding of treatment.

1.2.4 Methods of CTC detection

Since interest in the area of CTC research has grown over the last decade, there has been a surge in the development of new CTC detection platforms and methods of CTC isolation and characterisation. A review by Parkinson et al., summarises 43 CTC technologies that have been developed or are in the process of development for commercial use (Parkinson et al., 2012). In the past, CTC detection has relied on either imaged-based technologies, fluorescent-activated cell sorting (FACS) or polymerase chain reaction (PCR) methods to detect circulating free DNA (cfDNA). Only recently has it been achieved to combine these approaches into a single platform. Due to the rarity of CTCs in the peripheral blood of patients (as low as 1 cell/ 1 x 10^7peripheral blood mononucleocytes) there is a drive for platforms to be highly sensitive but there is also a requirement for them to be highly specific (Sleijfer et al., 2007). The ability to detect CTCs in such a high background of other cells usually leads to the integration of an enrichment technique as part of the sample processing. Methods of CTC enrichment can be categorised into those that are based on physical properties of the tumour cell versus

those that are dependent on biological properties; usually epitope characteristics. Alongside growing interest in the field of CTCs there has been an advent of new CTC detection platforms of which some of the most commonly used ones will be further discussed. When considering a technology platform for the detection CTCs, there are certain criteria that should be considered. Currently the Veridex CellSearch system is referred to as the gold standard for CTC detection and is the only system that has been clinically validated to stratify patients into groups that are considered to have a better or worse prognosis; however there are still limitations to this system that are beginning to be addressed and overcome by the advent of new technologies. Since there is potentially a wide clinical utility in the detection and characterisation of CTCs, the ability to integrate a detection platform into a clinical setting should be considered. In a review by Parkinson et al., the important clinical considerations of CTC assays are discussed, including pre-analytical variables (materials, sample collection, storage and shipping); analytic variables (sensitivity, specificity, reproducibility and controls); as well as post-analytical variables where multiple laboratories are involved in sample processing (inter-laboratory performance) (Parkinson et al., 2012). The numbers of variables that are involved in processing patient samples for CTC characterisation or enumeration are vast, highlighting the potential difficulties in implementing such a method clinically.

1.2.5 The CellSearch system

The CellSearch system is currently the only FDA-approved CTC detection technology in clinical use in the US for CTC detection in metastatic breast, prostate and colorectal cancer (Cohen et al., 2008, Cristofanilli et al., 2004, de Bono et al., 2008). In patients with metastatic breast cancer, metastatic sites were assessed using imaging and CTC measurements in the blood were taken before commencement of subsequent therapy (Cristofanilli et al., 2004). A threshold of 5 or more detectable CTCs was determined to be a worse predictor for OS and PFS (Cristofanilli et al., 2004). Furthermore, the same study showed that a high baseline level of CTCs was independently associated with worse OS (Cristofanilli et al., 2004).

The CellSearch processes 7.5 ml blood samples for the presence of CTCs defined by the expression of EpCAM, cytokeratin (CK) 4',6-diamidino-2-phenylindole (DAPI) and absence of haematopoietic marker common leucocyte antigen (CD45). Blood collected in CellSave tubes containing a preservative can be kept at room temperature (RT) for 96 h. This method utilises an automated CellTracks system whereby CK and EpCAM-positive cells are magnetically retained. Captured cells are permeabilised and fluorescently labelled enabling visualisation and enumeration using a CellTracks Analyser II (Janssen Diagnostics, 2016).

Although the CellSearch is the only CTC technology that has been implemented in a clinical setting - along with other CTC platforms available - it still has limitations. Firstly, the detection of CTCs is based on the criteria of the tumour cell being of epithelial origin and therefore does not address the problem of a heterogeneous CTC population. Since it has been hypothesised that cells metastasize by undergoing EMT, it is possible that they lose their epithelial phenotype and resort to a cell that would be more readily detected if a panel of mesenchymal markers was included in the positive selection. The CellSearch CTC isolation and detection technology was subject to extensive validation experiments to assess recovery; precision; intra-user, inter-user and inter-laboratory reproducibility; and the detection of CTCs in control groups. (Allard et al., 2004)

1.2.6 The Imagestream as a CTC detection platform

The Imagestream combines FACS with high resolution fluorescent, brightfield and darkfield microscopy; combining the speed and information produced by FACS with the detail and characterisation ability of microscopy. One of the major advantages of this technology is that whilst many other CTC platforms tend to rely on epithelial markers such as EpCAM or CK, the Imagestream has the ability to detect up to 10 different proteins of interest, providing they are structurally and spectrally different. It also has the additional feature extended depth of field (EDF) which enhances its use for applications such as fluorescent in situ hybridisation (FISH) or the counting of foci. The IDEAS software (Amnis, Seattle) has been specifically designed to complement the Imagestream technology. The system incorporates a virtual cell sorting feature which

enables the tracking of individual cells within populations and also has the ability to analyse the cells based on multiple features (Basiji et al., 2007).

A cell suspension is passed into a flow cell and focussed into a core through a sheath fluid which acts to separate out the cells and provide a clear background for imaging. Cells are illuminated by 5 excitation lasers which sit behind an LED array enabling brightfield imaging. Darkfield imaging is produced by an infra-red laser. Selected probes are detected by excitation with 5 different lasers (405 nm; 488 nm; 561 nm; 642nm and 785 nm). Three objectives are available for use (x20, x40, x60) which are used to collect light which is then passed through two arrays of dichroic mirrors – each set reflecting light of different bandwidths and producing 6 separate images, giving a total of 12 images per cell which are captured by a 12-bit charge coupled device (CCD) camera.

A comparison of the CellSearch system versus the Imagestream was conducted on the basis of CTC enumeration in spiking experiments (Lopez-Riquelme et al., 2013). The study concluded that both technologies were similar in terms of their performance of enumeration although the Imagestream was not as precise at detecting CTCs when in low numbers (1 or 10 CTCs) (Lopez-Riquelme et al., 2013). It is however important to note that this was not a direct comparison since the processing of the two samples relied on two different enrichment techniques: the CellTracks system was used for the CellSearch technology whereas EpCAM magnetic beads (MACS) were used prior to sample processing using the Imagestream. Furthermore different pan-CK antibodies were used for the two different technologies; suggesting that slight differences in CTC detection may not be as a result of the technologies but due to slight variations in the methods that were used during the processing of the sample.

While there is only one limited study comparing the Imagestream and the CellSearch system, the comparative advantages of each of the systems have been summarised below **Table 1.3**. CTC detection platforms should enable the characterisation of CTCs; based on cell morphology and biomarker expression. High resolution brightfield images that can be obtained using the Imagestream permit this – allowing 60x magnification whereas the CellSearch only enables 20x. As part of the characterisation of CTCs, the desired features of a CTC detection platform should include the freedom of the user to

implement biomarkers of interest to the cancer type or study. It is possible to implement biomarkers of interest into an assay design for the Imagestream, but currently the CellSearch is limited to epithelial biomarkers. Ideally the CTC detection platform should be highly sensitive and specific but also be practicable – enabling samples to be processed quickly. Only the CellSearch is currently FDA-approved; having undergone rigorous validation.

Optimal features	CellSearch	Imagestream
Independent of epithelial biomarkers	Dependent on EpCAM and CK	User may select antibodies of choice
Characterisation of cells	CTCs defined as EpCAM+ve/CK+ve/CD45-ve and nucleated	Interrogation of up to 10 cancer-specific biomarkers
Sensitive	Able to detect ≥1 CTC/ 7.5 ml of blood	Able to detect ≥1 CTC/ 8 ml of blood
Specific	Baseline levels of CTCs defined for different types of cancer. Biomarkers identify cells of epithelial origin	Able to implement cancer-specific biomarkers
Fast	Processing time ~24 h	Processing time ~4 h with enrichment
Clinically validated	FDA-approved	Not yet clinically validated
High resolution images	Magnification x20 available	Magnification x60 available

Table 1.3 Comparison of the Imagestream and CellSearch with the desired features of a CTC detection platform.

1.2.7 Alternative CTC isolation methods

One limitation with the detection of CTCs in the peripheral blood of patients is the volume of blood that is processed in many of the assays that have been developed. Despite the CellSearch system showing that there is a link between the number of CTCs detected and prognosis, there will always be a question of how representative a small aliquot of blood is of blood-borne metastasis and the metastatic process as a whole. Whereas most of the assays developed use 5-10 ml of blood, one such detection device – the GILUPI nanodetector – uses an in vitro approach to analyse the equivalent of 1500-3000 ml of blood (Saucedo-Zeni et al., 2012). The device consists of a gold-coated wire that is covered in a hydrogel coated with the anti-EpCAM antibody (Saucedo-Zeni et al., 2012). The device is inserted into a peripheral vein in the arm using a cannula and it is then left in situ for 30 min. CTCs are defined on the basis of CK positivity, CD45 negativity and nuclear staining (Hoeschst) (Saucedo-Zeni et al., 2012). Although this

technique may be considered to be more invasive, it is claimed to provide a similar level of discomfort to a regular blood draw. A limiting factor to this technique is that it relies on the capture of EpCAM-positive CTCs and would require clinical facilities for patients to undergo the procedure.

Another technique that has been proposed in order to evaluate a higher percentage of the patient's blood is to use the process of apheresis which entails processing 150 ml of buffy coat (an 8 ml blood sample usually yields ~1 ml of buffy coat) by filtering the blood of a patient and isolating the layer using density gradient centrifugation (Fischer et al., 2013). Although this method will result in an increased CTC yield, the clinical feasibility of such a method is questionable (Fischer et al., 2013).

To address the issue of having a method available to identify CTCs that is easily implemented in a clinical setting, several attempts have been made to create a CTC chip with the aim of producing a 'liquid biopsy'. One example is the microfluidic herringbone chip (HB-chip) which processes a 4 ml blood sample at rate of 1.5-2.5 ml/hour by subjecting it to a number of micro-vortices resulting in EpCAM-expressing cells being captured on a series of microposts (Stott et al., 2010). This chip has been used to test for the isolation of CTCs from patient with prostate cancer, after which cells were fixed and stained with prostate-specific antigen (PSA), CK and CD45 (Stott et al., 2010).

The dielectrophoresis (DEP) array (Silicon Biosystems) is another example of a microfluidic chip-based technique although it also combines the ability to image and isolate cells (Gascoyne et al., 2009, Gascoyne and Shim, 2014). The DEParray exerts dielectrophoretic forces around polarisable cells to form a 'cage'. Cells can be identified and imaged based on morphology or fluorescent markers. By altering the magnetic field, the cages can be moved by creating a magnetic field over a set of electrodes, enabling the isolation of single, viable cells (Gascoyne and Shim, 2014).

Vona and colleagues developed an assay that enables the separation of CTCs based on isolation of epithelial cells by size (ISET) (Vona et al., 2000). This process relies on passing whole blood through a set of filters that have 8 µm pores under a gentle vacuum. Filters can then be washed and collected cells can be analysed using

immunofluorescence, FISH and PCR following laser microdissection (Vona et al., 2000). Another CTC detection method that isolates CTCs on the basis of size is the Parsortix (Angle PLC, UK) (Parsortix PLC, 2016). The Parsortix is an automated, microfluidic device that captures CTCs based on the assumptions that CTCs are larger in size and less deformable in nature than haematopoietic cells (Parsortix PLC, 2016).

Another method of CTC isolation based on size is that of the micro- electro-mechanical system (MEMS). This consists of a polycarbonate filter that has pores etched into the surface at random intervals to capture cells that can then be lysed and assessed using PCR (Zheng et al., 2007). Size-based isolation methods are limited by the assumption that CTCs are notably larger than white blood cells (WBCs). Differences in size of CTCs compared to WBCs is one of the parameters that FACs relies on and methods of CTC isolation have been developed on this premise; however due to the heterogeneity of CTCs, there remains a possibility that the size of CTCs is similar to that of WBCs (Vona et al., 2000). Furthermore, size filtration methods may be limited in terms of recovery due to the blocking of pores by the agglutination of WBCs.

1.2.8 CTCs in HCC

So far there have been a limited number of studies into the presence of CTCs in HCC **Table 1.4**. In HCC, patients often present at an advanced stage at which point treatment options are limited. There may be a role for CTCs in in the earlier diagnosis of HCC; perhaps as a surveillance tool in patients with cirrhosis, if such a test that was inexpensive and sensitive could be developed. Currently the use of biomarkers used to monitor or stratify patients for treatment is limited to the use of AFP in combination with additional clinical parameters. In the UK, AFP is used as a stratification biomarker for patients in the assessment procedure for liver transplant. Serum AFP >1000 IU/ml is considered an absolute contra-indication for liver transplant in HCC patients in the UK due to the associated recurrence risk as outlined in research published by Duvoux and colleagues in France (Duvoux et al., 2012, Powell, 2014). Conversely, improved OS was observed in patients with elevated serum AFP levels (≥400 ng/ml or 1.5 times upper normal limit) in the REACH trial (Zhu et al., 2015). This was a phase III study of the use of ramucirumab versus a placebo in patients with advanced hepatocellular cancer as a

second line treatment to sorafenib (trial identifier: NCT01140347) (Zhu et al., 2015). The lack of clinical biomarkers in HCC indicates that validating HCC-specific CTC biomarkers could have a role in this context.

Method	Observations
CellSearch vs ISET (Morris et al., 2014)	Compared use of the CellSearch with the ISET for CTC detection Recovery rates were 28% (14/50) using the CellSearch and 100% (19/19) using the ISET GPC3 IHC was performed following CTC isolation with the ISET and compared to primary tumour biopsies 18/19 of the ISET samples had paired samples that were tested using the CellSearch system. Only 6/18 of these paired samples contained ≥1 CTC per 7.5 ml using the CellSearch system compared to 100% using the ISET technology.
CellSearch (Schulze et al., 2013)	CTCs were detected using EpCAM and CK-dependent CellSearch technology ≥1 CTCs were detected in 18/59 (30.5%) of HCC patient samples and 1/19 of the control group OS was shorter in the CTC-positive cohort (460 vs 746 days, p = 0.017) CTC number also increased with BCLC stage and AFP (>400 ng/mL)
ISET (Vona et al., 2004)	Microemboli were observed in 23/44 patients Presence correlated with raised AFP, diffuse tumours and PVT No association between tumour size or the number of nodules and number of CTCs Single CTCs were assessed for the presence of β-catenin mutations which were observed in 3/60 samples
RT-PCR (Wong et al., 1999)	AFP and albumin mRNA levels measured in 13 HCC patients compared to healthy control, patients with cirrhosis and spiked samples Determined that albumin-expressing cells were released intra-operatively whereas AFP-expressing cells were disseminated post-operatively Patients with consistently high AFP post-operatively died within 1 year following surgery
Positive magnetic selection based on asiaglycoprotein receptor expression (Xu et al., 2011)	Detection of circulating hepatocytes based on asiaglycoprotein expression Reported a minimum recovery rate of 61% based on spiking experiments using between 10 and 810 Hep3B cells Analysed isolated cells using immunofluorescence and FISH Reported the presence of CTCs in >80% of HCC patients tested and none were detected in healthy volunteers or patients with benign liver disease

Table 1.4 Summary of CTC studies in HCC. ISET=isolation by size of epithelial cell; CTC=circulating tumour cell; GPC3=glypican-3; IHC=immunohistochemistry; CK=cytokeratin; OS=overall survival; BCLC=Barcelona Clinic for Liver Cancer; AFP=alphafetoprotein; mRNA=messenger ribonucleic acid; RT-PCR=reverse transcriptase PCR; FISH=fluorescent in situ hybridisation

1.2.9 Circulating tumour DNA

In addition to investigations into the role of CTCs, studies into circulating free nucleic acids (cfDNA/cfRNA) also termed circulating tumour DNA (ctDNA) have been carried out in attempt to determine if the blood can be used as an indicator of tumour burden and in attempt to decipher the process of metastasis. ctDNA is DNA circulating in the peripheral bloodstream that is thought to be derived from the primary tumour. Initially this was based on the observation that levels of ctDNA are increased in individuals with cancer compared to healthy volunteers (Leon et al., 1977).

Many methods to detect ctDNA are heavily reliant on PCR. Although PCR-based methods are highly sensitive and relatively inexpensive, initial methods were not always highly specific and are subject to contamination by leucocytes (Smirnov et al., 2005, Vona et al., 2000). Such methods were also dependent on there being no circulating DNA from normal tissues although in order to compensate for this, many studies include a control group and looked at differences above the threshold of the control group. Improved PCR-based technologies such as allele-specific PCR and mutation-specific PCR improve the reliability of such methods. Specificity can be improved by investigating characteristics consistent with that of tumour DNA such as mutations in oncogenes, tumour-suppressor genes, allelic gain or loss and strand instability (Anker et al., 1999).

In HCC, a study carried out by Wong and colleagues demonstrated that p16 methylation status could be detected in tumour tissues from 16/22 patients (73%) and out of those patients 13/16 (81%) had p16 methylation detected in their serum/plasma (Wong et al., 1999). A more recent study of 142 plasma samples from patients with HCC, cirrhosis or chronic viral hepatitis quantified ctDNA using PCR to amplify the human telomerase reverse transcriptase (hTERT) concluded that although the concentration of ctDNA may not be useful as a HCC diagnostic biomarker, however in viral HCC it may be a useful prognostic indicator (Piciocchi et al., 2013). The concentration of ctDNA increased in patients with multinodular HCC (>3 nodules) and tumour size (p=0.002) and hTERT

levels (p=0.02) were independent predictors of prognosis although there were no significant associations between ctDNA and age, sex, AFP, tumour grading or Child-Pugh score (Piciocchi et al., 2013).

One of the disadvantages of detecting ctDNA as opposed to CTCs is that the origin of the ctDNA cannot be determined. Although without the use of a viability marker in CTCs it cannot be determined whether or not the cells are capable of forming extra-hepatic metastases, however by detecting ctDNA it cannot be known whether the DNA detected is due to the presence in cells, or whether it is an artefact from cells that have undergone cell death. There still remains a difficulty in detecting low amounts of mutant DNA amongst a high background of wild type (wt) DNA. Furthermore they do not enable conclusions about cell morphology to be made (Vona et al., 2000). A recent study by Bettegowda and colleagues demonstrated that ctDNA was detected in cases where CTCs were not detected implying that ctDNA and CTCs are separate entities (Bettegowda et al., 2014).

1.3 HCC biomarkers

Biomarkers are defined as “objectively measured indicators of biological processes or response to a therapeutic intervention.” (Firestein, 2006). As stated previously, there is no single biomarker that can be used for prognosis in HCC. The EASL-EORTC guidelines state that in order for a biomarker to be implemented into the clinical management of HCC it should “(1) demonstrate prognostic prediction in properly powered randomised studies or in training and validation sets from cohort studies; (2) demonstrate independent prognostic value in multivariate analysis, including known clinic-pathological predictive variables; and (3) demonstrate confirmation of results using the same technology in an external cohort reported by independent investigators.” (Simon et al., 2009, EASL-EORTC, 2012).

Current methods of CTC identification tend to rely on the definition that a CTC derived from an epithelial tumour will be nucleated and express epithelial markers – commonly EpCAM and cytokeratin in addition to being negative for the haematopoietic marker CD45. Some studies also include morphological characteristics of the cell as an important determinant of the CTC definition – for example size - however it has been noted that there is great heterogeneity within the morphological characteristics of CTCs. In order to positively differentiate CTCs from other circulating cells – for example circulating endothelial cells (CECs), there is a requirement to include markers that are sensitive and specific to hepatocellular carcinoma. Currently there is no single marker that is able to positively identify all cases of HCC. Whilst AFP remains to be the most widely clinically used serum biomarker, not all HCCs are AFP positive and serum elevation of AFP is also indicative of benign inflammatory liver conditions or during liver repair.

In order to detect CTCs in patients with HCC with a confident degree of sensitivity and specificity it is likely that a panel of biomarkers will need to be used. In this research, the expression on CTCs of a combination of markers was investigated. This included the epithelial biomarkers EpCAM and CK; the clinically implemented HCC biomarker AFP

and several exploratory biomarkers that may be used to guide therapy including: glypican-3 (GPC3), sulfatase 2 (SULF2) and DNA-dependent protein kinase (DNA-PK). The common leucocyte antigen CD45 was used to distinguish WBCs from CTCs.

1.3.1 Cytokeratin

CKs are intermediate filaments that are considered to have a regulatory role in cell migration and invasion (Barak et al., 2004, Ding et al., 2004). There are over 20 different CKs, although 8, 18, and 19 are the most commonly expressed in epithelial cells (Barak et al., 2004). The expression of CK is retained in malignant cells, making them a choice candidate for the recognition of tumour cells (Barak et al., 2004). Cytokeratins have been used as a serum biomarker in patients with epithelial cancers. In the field of CTCs, CKs are commonly used to form part of the CTC definition for epithelial neoplasms. In HCC the expression of CK-7 and -19 may be expressed in hepatic progenitor cells and cholangiocytes but not in normal hepatocytes (Durnez et al., 2006). Furthermore, the expression of CK-19 correlated with raised serum AFP and a higher rate of recurrence following transplantation (Durnez et al., 2006). CK-19 was initially used to differentiate between HCC and cholangiocarcinoma (Balaton et al., 1988). Ding and colleagues reported increased protein expression of CK-19 in HCC cell lines with higher metastatic potential and in patient samples using immunohistochemistry (IHC) (Ding et al., 2004). CK-19 expression was associated with more poorly-differentiated tumours and has been reported as being indicative of poor prognosis in HCC; arising as a result of expression of progenitor cell features or a mixed hepatocellularcholangio phenotype (Ding et al., 2004, Roskams, 2006, Razumilava and Gores, 2013).

1.3.2 EpCAM

EpCAM is a 40 kDa single-pass type I membrane protein encoded by the TACSTD1 gene (Yamashita et al., 2007). It is classed as a hepatic stem cell marker that is expressed on the majority of hepatocytes in embryonic liver and it a direct transcriptional target of the wnt/β-catenin pathway (Yamashita et al., 2007). EpCAM is expressed in the

majority of neoplasms that are of epithelial origin; though in HCC this overexpression is mirrored by elevated serum levels which are detected in 60-70% of patients (Balzar et al., 1999, Yamashita et al., 2008).

There is conflicting evidence for the utility of EpCAM as a biomarker in many different types of cancer and this extends to its role in HCC. Mature hepatocytes do not express EpCAM although its expression is highly elevated in pre-malignant hepatic tissues and a subset of HCC (Yamashita et al., 2007). A study carried out by Gorges et al., who performed spiking experiments using the AdnaTest – an EpCAM –positive selection technique - alongside gene expression of CTCs to look at changes during EMT noted that EpCAM expression was downregulated (Gorges et al., 2012).

It has been proposed that EpCAM-positive cells may represent a more phenotypically aggressive proportion of the CTC population. Yamashita and colleagues categorised HCC cases into four sub-types based on the expression of EpCAM and AFP. Results of a HCC cDNA microarray, oligonucleotide microarray and IHC were analysed to explore the differential expression of genes between the groups of HCC based on EpCAM and AFP expression (Yamashita et al., 2008). In total there were 71 differentially expressed genes between EpCAM-negative and EpCAM- positive cases of HCC. Furthermore each of the four categories displayed a different pattern of gene expression which correlated to that of hepatic cell lineages: EpCAM-positive cases of HCC displayed a unique molecular signature consistent with hepatic progenitor cells whereas the genetic profile of EpCAM-negative HCC cases was similar to that of mature hepatocytes (Yamashita et al., 2008). The same research group went on to further characterise the subtypes of HCC cells based on their AFP and EpCAM expression; concluding that EpCAM and AFP positive cells had a hepatic stem/progenitor cell phenotype with activated wnt/β-catenin signalling (Yamashita et al., 2009). These cells had increased tumour initiating capabilities determined by the results of subcutaneous injection of EpCAM positive cells in a non-obese severe combined immunodeficient (NOD/SCID) mouse model in comparison to EpCAM negative cells (Yamashita et al., 2009). Consistent with this data are results of a study carried out by Sun and colleagues who injected EpCAM-positive and EpCAM-negative cells into two different groups of NOD/SCID mice (Sun et al.,

2013). The same study also investigated the prognostic relevance of the detection of EpCAM positive cells in the blood of patients both pre- operatively and post-operatively. This was carried out using the CellSearch system (Veridex) on three groups of patients that were classified as healthy individuals (n=10); patients with benign liver disease (n=5) and patients with HCC (n=123) (Sun et al., 2013). The study concluded that a pre-operative measurement of ≥2 EpCAM positive CTCs was indicative of tumour recurrence, even in cases of HCC where AFP levels were ≤400 ng/ml (Sun et al., 2013).

1.3.3 AFP

AFP is a glycoprotein that is first produced in the yolk sac and expressed in the embryonic liver (Arrieta et al., 2007). Post-natal production of AFP decreases rapidly and AFP expression is not a normal characteristic of adult liver. AFP is detected normally in the serum of healthy individuals at a level of ≤20 ng/ml although this varies slightly between different ethnicities (Zhou et al., 2006). AFP elevation (>20 ng/ml) is associated with the onset of HCC and is currently the most widely used clinical marker. However AFP elevation cannot be used singly as a diagnostic marker of HCC due to serum levels above the normal range also being indicative of CLD such as infection with HCV which is considered to be a pre-malignant condition. It is generally estimated that AFP elevation occurs in approximately 50-70% of cases of HCC and it has been suggested that AFP levels are representative of tumour burden, though some reports in the literature quote the incidence of elevated AFP in HCC is even higher than this (Johnson, 1999, Kawai et al., 2001, Zhou et al., 2006). Measurement of serum AFP has been used to monitor response to treatment (Johnson, 1999). Studies have shown that dramatically elevated AFP pre-treatment is predicative of prognostic outcome in patients receiving a liver transplant or TACE treatment (Yokoo et al., 2004, Samad et al., 2012, Wang et al., 2012b). Yokoo and colleagues showed the protein expression of AFP in 11 liver cancer cell lines (Huh-1 and -7; JHH-7 and -5; HepG2; HT17; Hep3B; Li-7; PLC/PRL/5; KIM-1; KYN-2) (Yokoo et al., 2004).

cDNA microarray analysis of 5 hepatoma cell lines (PLC/PRF/5HepG2, Hep3B, Huh-7 and -6,) showed that AFP-producing cell lines have a shared genetic profile but more specifically, cell lines with higher AFP expression (HepG2, Hep3B and Huh7) are more closely associated in terms of the expression of sub-sets of oncogenes and genes that are associated with apoptosis, cell-cell interaction and cell cycle (Kawai et al., 2001). A limitation with the use of AFP as a biomarker is that it is often only expressed in the later stages of HCC and in some cases it is not expressed during any stage of the disease course, indicating that from a diagnostic aspect its detection is of limited value (Lopez, 2005).

1.3.4 Glypican-3 (GPC3)

Located at Xq26, the GPC3 gene encodes a phosphatidylinositol-anchored plasma-membrane heparan sulfate proteoglycan (HSPG) that exists in four isoforms of which isoform 2 is the most commonly expressed (Ho, 2011). GPC3 is expressed in foetal liver tissue but repressed following DNA methylation in adults (Sakamoto et al., 2010). HSPGs mediate and modulate signalling with growth factors and chemokines such as IGF-2 and Wnt complexes; regulating proliferation and cell survival (Suzuki et al., 2010, Ho, 2011, Yao et al., 2011). The roles of GPC3 on the proliferation and invasive potential of cells appears to vary depending on expression levels (Ho, 2011, Kwack et al., 2006).

Microarray data has shown levels of GPC3 mRNA are significantly increased in patients with HCC compared to healthy controls or patients with CLD (Zhu et al., 2001). Furthermore, it has been identified that GPC3 mRNA levels are higher in HCC cases that are considered to be more successfully resected and have a less aggressive tumour phenotype compared to tumours exhibiting the opposite properties (Zhu et al., 2001). The use of GPC3 as a diagnostic serum marker has been investigated concluding that the serum concentration of GPC3 is not correlated with AFP serum concentration and that the use of both markers may increase the sensitivity of diagnostic testing using HCC tumour markers in the blood of patients (Capurro and Filmus, 2005, Nakatsura et al., 2003). However a limitation associated with use of GPC3 as a diagnostic marker is that

despite being highly specific, sensitivity is low (40%), detecting GPC3 in only 16/40 of HCC patients (Nakatsura et al., 2003).

Overexpression of GPC3 in HCC has suggested its potential as a therapeutic target, leading to the development of a humanised monoclonal antibody against GPC3 (GC33) (Zhu et al., 2013). Phase II clinical trials have recently been completed for the use of GC33 concluding that it is well tolerated (Zhu et al., 2013).

1.3.5 Sulfatase 2 (SULF2)

Sulfatase 1 (SULF1) and sulfatase 2 (SULF2) are cell-surface secreted endosulfatases that catalyse the 6-O desulfation of HSPGs (Lai et al., 2010, Rosen and Lemjabbar-Alaoui, 2010). Functioning as a tumour suppressor, SULF1 has opposing activity to SULF2 due to the abrogation and activation of fibroblast growth factor (FGF) signalling pathways respectively (Lai et al., 2010, Yang et al., 2011). SULF2 exerts an oncogenic effect in HCC: increasing cell growth and migration in vitro as well as Hep3B SULF2 over-expressing cells increasing tumour grow in xenografts in mice (Lai et al., 2010). Desulfation of HSPGs (e.g. GPC3) releases growth factors, increasing their availability to receptors, resulting in the activation of downstream signalling pathways including FGF and Wnt/frizzled/β-catenin (Lai et al., 2010). SULF2 expression is increased in approximately 60% of HCCs and has been shown to up-regulate GPC3 at the protein level. In 11 HCC cells lines, 8 positively expressed SULF2 (HepG2, Huh-7, SkHep1, SNU182, SNU387, SNU423, SNU449 and SNU475) and immunocytochemistry revealed that SULF2 co-localises with GPC3 (Lai et al., 2010). Furthermore, knockdown of GPC3 led to a decrease in FGF2 binding suggesting it plays an important role mediating signalling through FGF-activated pathways (Lai et al., 2010). Clinically high SULF2 mRNA expression correlated with worse OS and PFS (Lai et al., 2010).

1.3.6 DNA-PK

DNA protein kinase (DNA-PK) is a protein kinase involved in non-homologous end joining (NHEJ) of lethal DNA double strand breaks (DSBs) (Collis et al., 2005). The catalytic subunit of DNA-PK (DNA-PKcs) is recruited to the site of a DSB and activated by autophosphorylation following recognition and binding of the Ku70/80 heterodimer to the broken DNA strands (Chan et al., 2002, Collis et al., 2005). The nuclease Artemis is also recruited and the DNA-PK complex is able to align the strands of DNA which are then joined back together through the DNA ligase IV/ X-ray cross complementation group 4 protein (XRCC4) complex (Collis et al., 2005).

Deregulation of DNA-PK has been identified in multiple solid tumours including colorectal cancer (Hosoi et al., 2004), gastric cancer (Lee et al., 2005) and haematological cancers including acute lymphoblastic leukaemia (ALL) and chronic lymphoblastic leukaemia (CLL) (Holgersson et al., 2004, Willmore et al., 2008) as well as others. It was hypothesised that inhibition of DNA-PK may result in sensitisation of cells to ionising radiation since cells would be unable to repair DNA DSBs via the NHEJ pathway.

In HCC, total DNA-PK and DNA-PKcs expression and activity was increased in tumour tissue from patients undergoing surgical resection compared to surrounding liver and normal liver tissue (Evert et al., 2013). Patients undergoing partial hepatectomy surviving less than 3 years had higher levels of DNA-PK protein expression and activity compared to those surviving more than 3 years (Evert et al., 2013).

Previous work in Newcastle, carried out by Cornell and colleagues, further explored the role of DNA-PK in HCC (Cornell et al., 2015). Increased DNA-PK copy number and protein expression levels were reported in association with advanced HCC stage. Inhibition using a selective inhibitor of DNA-PK (NU7441) potentiated ionising radiation and chemosensitised HCC in cell line and murine models (Cornell et al., 2015). DNA-PK expression levels assessed using IHC in HCC patient tissue biopsies suggested that it may be an independent predictor of response to TACE, with patients with low DNA-PK

expression in tissue biopsy samples having a longer time to progression (TTP) (16.9 months vs 4.5 months) and a longer median survival (35 months vs 9.9 months) (Cornell et al., 2015). This data suggests DNA-PK may be a useful biomarker to stratify HCC patients for treatment with TACE (Cornell et al., 2015).

1.3.7 c-Met

c-Met – mesenchymal epithelial transition factor - is a receptor tyrosine kinase that binds its ligand the hepatocyte growth factor (HGF) which is secreted by stellate cells leading to activation of multiple signalling pathways (Llovet and Bruix, 2008). Such signalling pathways include PI3K-Akt, ERK/MAPK, Crk/Rap and Rac/Pak (Birchmeier et al., 2003). Under normal physiological circumstances, HGF and c-Met are required for embryonic viability, normal liver formation and regeneration in response to injury (Bladt et al., 1995, Schmidt et al., 1995). In cancer, altered MET activity as a result of mutations or overexpression have been associated with increased tumour growth, evasion of apoptosis, angiogenesis and metastasis, contributing to a poorer prognosis (Farazi and DePinho, 2006, Migliore and Giordano, 2008, Organ and Tsao, 2011, Wang et al., 2001).

In HCC functional genomic cluster analysis demonstrated that aberrant c-MET activity was associated with a poor prognosis and indicative of an aggressive phenotype (Kaposi-Novak et al., 2006). In a tetracycline-inducible Met transgenic mouse model, it was found that Met was activated by cell adherence in a ligand-independent fashion, leading to the development of liver tumours in mice that regressed following inactivation of the MET transgene (Wang et al., 2001). Another study showed that binding of HGF to Met leads to phosphorylation of beta-catenin leading to its activation and translocation to the nucleus in hepatocytes (Monga et al., 2002).

Due to the downstream effects of HGF/c-Met binding and its overexpression in cancers - including HCC, attempts have been made for the therapeutic targeting in cancer using monoclonal antibodies against HGF/c-Met, selective c-Met tyrosine kinase inhibitors (TKIs) and multi-TKIs (Goyal et al., 2013, Matsumoto et al., 2008, Migliore and Giordano,

2008, Santoro et al., 2013b, Wang et al., 2001). In a study of 21 HCC patients with HCC staged as BCLC B/C and Eastern cooperative oncology group (ECOG) performance status 0-1, a phase 1b trial of tivantinib (ARQ197) was designed (Santoro et al., 2013b). Tivantinib is a selective competitive inhibitor of c-Met that had been trialled in other cancers, though it was not known whether or not the drug would further impair liver function in patients with HCC (Santoro et al., 2013b). Initial studies showed that there was no drug-related worsening of liver function leading to further trialling of tivantinib in a randomised control trial (RCT). Although there was no significant differences in outcome between the two groups of randomised patients, a subanalysis suggested that in patients with high tumour Met expression, tivantinib resulted in a longer TTP compared to those receiving a placebo (2.7 months vs 1.4 months) (Santoro et al., 2013a). The study similarly suggested Met levels predicted a response to tivantinib in terms of OS (p=0.039) and that Met was an independent prognostic factor for previously treated patients with HCC (Santoro et al., 2013a). The use of tivantinib in patients with high tumour MET expression - determined by IHC - showed promising results in phase 2 RCT studies (Giordano and Columbano, 2014, Rimassa et al., 2015).

1.3.8 pERK as a surrogate marker of activation of the Ras/Raf/MAPK pathway

Evidence of aberrant Ras/ERK activity as a result of upregulation, genomic amplification or methylation of constituents of the pathway has been identified in HCC (Llovet and Bruix, 2008, Newell et al., 2009). Phosphorylated ERK (pERK) can be used as a surrogate marker for the activation of the Ras/Raf/MAPK pathway since pERK leads to the activation of transcription factors responsible for genes involved in the regulation of proliferation and survival (Newell et al., 2009). In HCC compared to non-tumour tissue there was decreased sprout-related protein with Ena/vasodilator-stimulated phosphoprotein homology-1 domain (Spred) which is a physiological inhibitor of Ras/Raf-1/ERK pathway (Yoshida et al., 2006). Overexpression of ERK has been associated with disease progression in HCC (Ito et al., 1998). Furthermore, Spred expression inversely correlated with invasion and metastasis indicating that inhibition of the Ras/Raf-1/ERK pathway may be a potential therapeutic target in HCC (Yoshida et al., 2006).

Previous studies within the hepatology research group at Newcastle have indicated increased activation of the RAS signalling pathway in a murine model of carcinogenesis. A C3H/He murine model fed the American lifestyle diet (ALIOS) was shown to have an increased mean number of tumours compared to the DEN control group as well as developing histopathological features of steatohepatitic-HCC (SH-HCC) at 1 year including: microvesicular steatosis, ballooning and inflammation of hepatocytes (Whitehead et al., 2015). Additionally, there was increased expression of AFP, GPC3, neighbour of punc-E11 (NOPE), inducible nitric oxide synthase (iNOS) and tumour necrosis factor α (TNFα) at the mRNA level in liver tumours compared to normal liver tissue; indicating that this murine model could be a promising animal model for the study of human MetS associated HCC. Initial exploration into rat sarcoma viral oncogene homolog (RAS) pathway activation in this murine model was carried out. There was increased expression of pERK in tumour tissue compared to non-tumour tissue at the protein level assessed by Western analysis and measured using densitometry (99.8 ± 26.0 compared to 5.5 ± 0.6, p=0.004) (Whitehead et al., 2015). Furthermore, HRAS mutations at codon 61 were found to be present in 5/20 (25%) of mice confirmed by sequencing (Whitehead et al., 2015).

Results demonstrating increased activation of the RAS signalling pathway led to the hypothesis that RAS signalling may also be activated in human MetS-associated HCC. Paraffin-embedded needle biopsies from 20 HCC patients were assessed for pERK expression using IHC. Expression was digitally quantified using an algorithm developed using Aperio Imagescope. Minimal expression was defined as <5% tumour nuclei positive for pERK, excess as >50% pERK positive nuclei. 3/20 (15%) MetS-HCC patients had minimal pERK expression, 11/20 (55%) had moderate expression and 6/20 (30%) had excess expression (Whitehead et al., 2015).

Refametinib (BAY86-9766), a MEK1/2 inhibitor is currently in clinical trials (trial identifier: NCT01915589) for the treatment of HCC patients with unresectable or metastatic disease with a known KRAS or NRAS mutation confirmed by a beads, emulsification, amplification, and magnetic technology, sensitive mutation detection (BEAMing) plasma test (Schmieder et al., 2013).

1.3.9 Common leucocyte antigen - CD45

The inclusion of a haematopoietic marker enables differentiation between cells of interest and WBCs. Often CD45 is used to negatively select for CTCs in a sample through the means of WBC depletion. Similar to the method for positive EpCAM selection, antibodies against CD45 are conjugated to magnetic beads. This method does not rely on the tumour cells having a specific characteristic and depletion methods that have implemented this system have achieved relatively high recovery rates of 46-62% (Lin et al., 2013). A possible disadvantage of this method is that it may deplete CTCs that are surrounded by and interacting with WBCs.

1.4 The role of immune the immune system and tumour microenvironment in HCC

In HCC it is not only tumour cells that are considered to contribute to the process of hepatocarcinogenesis and predict outcome. In 90% of cases, HCC is developed on a background of inflammation (Nikolaou et al., 2013). Activated immune cells infiltrate the liver and contribute to the tumour microenvironment. In the final section of this literature review, the immune system and the tumour microenvironment in HCC will be discussed.

1.4.1 Components of blood

Constituents of blood include: plasma, leucocytes, erythrocytes and platelets. Blood cells are derived from multipotent haematopoietic stem cells and mature to form differentiated cells during the process of haematopoiesis **Figure 1.2**. Erythrocytes are the most abundant blood cell type, making up between 93-96% of blood cells. Erythrocytes - derived from myeloid lineage - are the oxygen transporting cells in the blood and are therefore rich in haemoglobin. Under normal physiological conditions, leucocytes make up just 0.1-0.2% of blood cells. They have an important role in the immune system and the individual roles of each cell type will be discussed in **Section 1.4.2**. Platelets are anuclear cells derived from megakaryocytes that are required for the maintenance of haemostasis (Semple et al., 2011). Platelets contribute to 4-7% of blood cells. During normal physiological conditions, platelets circulate in the blood in a quiescent state but they may be activated in response to vessel injury, or as part of their role in inflammation (Semple et al., 2011, Stanger and Kahn, 2013).

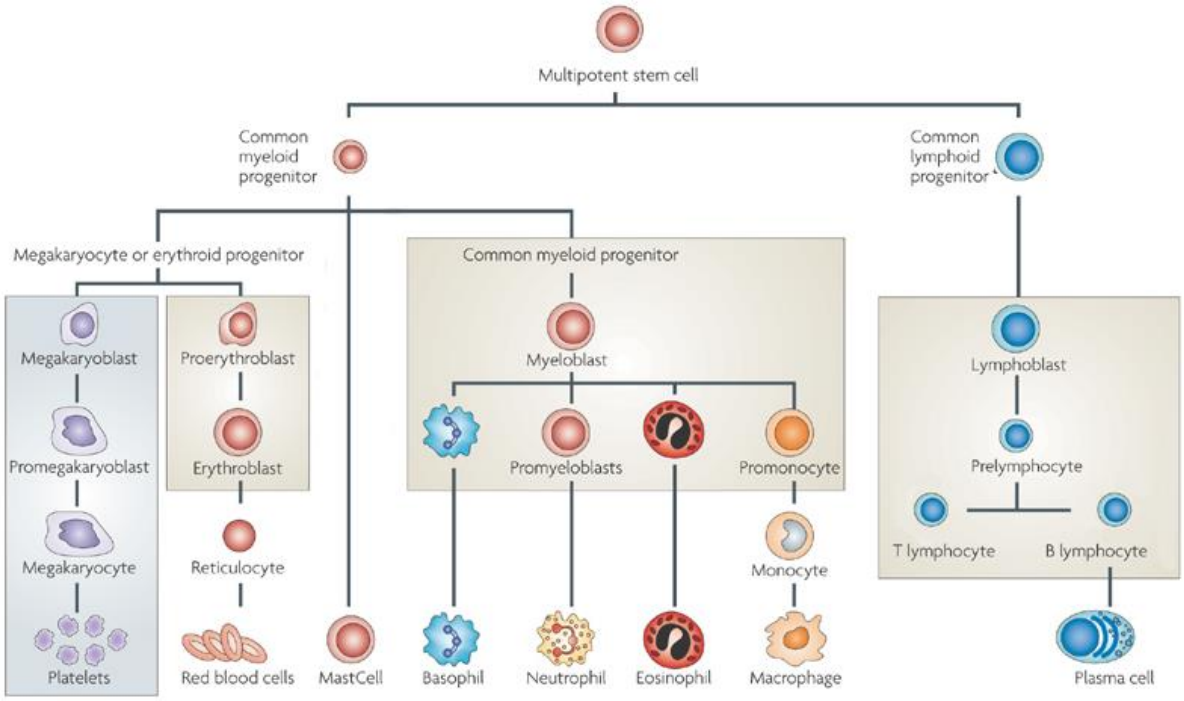


Figure 1.2 Haematopoiesis demonstrating the derivation of cells present in the blood. Adapted from (Ramsay and Gonda, 2008).

1.4.2 The immune system

The immune system is broadly divided into cells and molecules which either make up the innate response or the adaptive response **Table 1.5-Table 1.6** and **Figure 1.3** (Dranoff, 2004, Peakman, 2009). The innate response is an unspecified but immediate form of first-line defence against pathogens which is usually responsive within hours (Male, 2006). Adaptive immunity is a slower (within days) but specific response dependent on antigen expression (Male, 2006). Following first exposure to the antigen, there is clonal expansion of cells specific to that antigen and an immunological memory is built up so that on second presentation of the same antigen a faster response is elicited (Male, 2006). Additionally, chemical messengers such as chemokines and cytokines contribute to the immune response. Chemokines are a family of cytokines that are associated with chemotaxis of cells; their main roles include regulation of lymphoid organ development and T cell differentiation but they have also been recognised mediators of tumour metastasis (Keeley et al., 2011). Cytokines are proteins or glycoproteins that are

important cell signalling molecules that orchestrate coordination and function of haematopoietic cells and inflammatory cells during an inflammatory response (Peakman, 2009, Male, 2006).

Cells and molecules associated with the innate immune response		
Classification		
Granulocytes	Neutrophils	Phagocytose and kill microorganisms Release anti-bacterials
	Eosinophils Basophils Mast cells	Release pro-inflammatory mediators
Mononucleocytes	Monocytes Macrophages	Ingest and kill bacteria Release pro-inflammatory mediators Antigen presentation
Dendritic cells	Myeloid dendritic cells Plasmocytoid dendritic cells	Migrate to inflammatory sites Antigen presentation Release interferons
Lymphoid cells	Natural killer cells	Lyse infected cells Kill target cells spontaneously Secrete cytokines Properties associated with both innate and adaptive function
Complement proteins	Cascade of >40 proteins	Promote inflammation Recruit cells Kill targeted cells Remove immune complexes

Table 1.5 Cells of the innate immune system. Adapted from (Peakman, 2009).

Cells associated with the adaptive immune response		
Cell Classification		
T-lymphocytes	T-lymphocytes	Organise killing of microorganisms Activate macrophages
	Helper T lymphocytes	Organise killing of parasites via eosinophil recruitment
	Cytotoxic T-lymphocytes	Ill-defined Recruit cells
	T regulatory cells	Regulate inflammation
B-lymphocytes		
Lymphoid cells	Natural killer cells	See**Table 1.4**
γδ T cell	γδ T cell	Secrete IL-17/ IFNγ Mediate rapid response to specific pathogens similarly to NK cells Paradoxical role in cancer

Table 1.6 Cells of the adaptive immune system. Adapted from (Peakman, 2009, Vantourout and Hayday, 2013, Silva-Santos et al., 2015, Vivier et al., 2011).

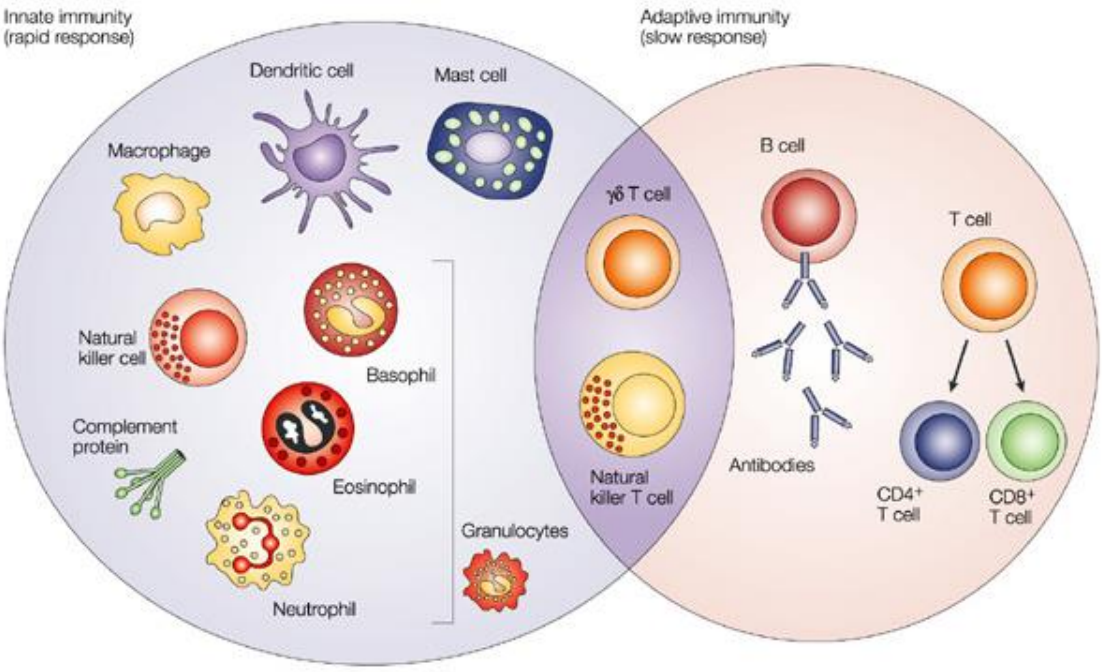


Figure 1.3 Cells and molecules of the innate and adaptive immune responses (Dranoff, 2004).

1.4.3 Immune surveillance in the liver in normal physiological conditions

In order to prevent infection from blood-borne infection, the liver performs immune surveillance under normal physiological conditions. The anatomy and blood supply to the liver aids in the role of immune surveillance. Additionally, the liver is populated with resident immune cells that occupy the liver parenchyma and vasculature including: Kupffer cells, hepatic stellate cells, dendritic cells and lymphocytes (Jenne and Kubes, 2013). Located in the vasculature, Kupffer cells adhere to liver sinusoidal endothelial cells (LSECs) and are densely populated in the liver, accounting for approximately 80-90% of the total macrophages in the body (Jenne and Kubes, 2013). Of the residing lymphocyte population in the liver, there is a high proportion of innate natural killer (NK) cells and NK T-cells (NKT cells) compared to the peripheral blood (Racanelli and Rehermann, 2006). NK and NKT cells have roles implicated in tumour surveillance (Racanelli and Rehermann, 2006). Parenchymal liver cells can also contribute to the

immune surveillance of the liver. This may be via the production of cytokines which have been associated with hepatocarcinogenesis – for example hepatocytes and Kupffer cells produce cytokines including interleukin-1 (IL-1), TNFα and interleukin-6 (IL-6) (Budhu and Wang, 2006). Liver endothelial cells are present in the sinusoidal lumen and are imperative for pathogen detection and capture; whilst hepatocytes have been associated with secretion of inflammatory cytokines (Rowell et al., 1997). Activated macrophages are the main source of cytokines though they may be secreted by any nucleated cell (Coussens and Werb, 2002).

1.4.4 Immune response in HCC

An exacerbated immune response is characterised by the infiltration of immune cells; a common feature of most neoplastic lesions at varying degrees (Hanahan and Weinberg, 2011). Innate immune cells recognise cellular damage, pathogen invasion and metabolic stress (Bieghs and Trautwein, 2013, Gorham, 2007). This is characterised by dysregulated cytokine production mainly consisting of: tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and transforming growth factor β (TGFβ) which collectively activate downstream signalling pathways resulting in pro-/anti-apoptotic responses, acute-phase protein synthesis and altered hepatocyte proliferation (Bieghs and Trautwein, 2013). The adaptive immune response is characterised by the activation of lymphocytes and macrophages. There is a resident population of adaptive immune cells in the liver which is comprised mostly of T cells (~50%) and NK cells (~33%) (Gorham, 2007). Adaptive immune cells have been recognised as having both pro-tumorigenic and anti-tumourigenic roles in HCC (de Visser et al., 2006, Schneider et al., 2012). One study that utilised a N-nitrosodiethylamine (DEN) model of hepatocarcinogenesis in Rag1-/- mice (lacking mature T- and B-cells) showed that tumour growth occurred earlier and there was an increased number of tumours compared to wild type mice; in this instance indicating that T- and B-lymphocytes offer an protective anti-tumourigenic mechanism (Schneider et al., 2012). Gene microarray studies of 139 HCC human samples - performed by the same group – showed that unsupervised clustering gave rise to two groups of patients based on the differential expression of genes associated with T-cells and B-cells and that prognosis was different between the two groups of patients. It

should be noted that the innate and adaptive immune systems do not act independently – there is interplay between both systems.

1.4.5 The role of inflammation in cancer

The immune system has a paradoxical role in many types of cancer, including HCC. Initially it was thought that the immune system would offer a protective role against tumour cells by recognising and destroying them. However, it is now also considered that the immune system can have a pro-tumorigenic effect; enabling tumour cells to evade immune destruction as well as contributing to an inflammatory response adding to the pro-tumourogenic properties of the tumour microenvironment (Hanahan and Weinberg, 2011). Historically, the first link between inflammation and cancer was made by Rudolf Virchow in 1863 who documented infiltrating leucocytes in cancerous tissue and associated this with cancer development in the presence of chronic inflammation (Balkwill and Mantovani, 2001). Recently, the importance of the role of the immune system in the development and progression of cancer has been recognised. In an updated version of Hanahan and Weinberg's "Hallmarks of cancer: the next generation", inflammation was identified as an enabling characteristic in tumorigenesis (Hanahan and Weinberg, 2011).

Chronic inflammation can pre-dispose individuals to cancer (e.g. inflammatory bowel disease is associated with the development of colorectal cancer and inflammation of the prostate is associated with an increased risk of prostate cancer) (Mantovani et al., 2008, Shacter and Weitzman, 2002). Furthermore, the link between the use of anti-inflammatory drugs and a reduced cancer risk has emerged: with the use of non-steroidal anti-inflammatory drugs (e.g. aspirin) resulting in a reduced cancer risk in patients with oesophageal, gastric and colorectal cancers (Thun et al., 1993, Thun et al., 1991). More recently, studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) – which inhibit cyclooxygenase 2 (COX2) and hence the signalling of prostaglandins – also reduce the risk of breast and lung cancer; however there has been associated cardiac associated toxicity with the long term use of NSAIDs (Ulrich et al., 2006).

The link between inflammation and cancer can be divided into two separate but interconnected pathways: the intrinsic and the extrinsic pathway (Mantovani et al., 2008). The extrinsic pathway is defined as that which occurs in response to chronic inflammation, whereby transcription factors are activated leading to the secretion of chemokines, cytokine and prostaglandins which leads to the recruitment of inflammatory cells (Mantovani et al., 2008). The intrinsic pathway follows the process of events characterised by activation of oncogenes (Mantovani et al., 2008). One of the most common examples in human cancers are the mutations of the RAS family of oncogenes, resulting in activation of the RAS/RAF signalling pathway, leading to the production of inflammatory cytokines and chemokines (Sparmann and Bar-Sagi, 2004). Pro-tumourogenic effects of downstream Ras signalling include proliferation, angiogenesis, metastasis and modulation of the tumour microenvironment (Pylayeva-Gupta et al., 2011).

1.4.6 The tumour microenvironment

The tumour microenvironment is made up of the tumour cells, immune cells, associated stroma and vasculature as well as the signalling and interactions that take place between these features. The concept is derived from Paget's 'seed and soil' hypothesis whereby it was suggested that specific environmental conditions were required in order for tumour cells to grow (Weber and Kuo, 2012). The tumour microenvironment is not static and it changes over time throughout tumour development (Quail and Joyce, 2013). It has been suggested that both innate and adaptive immune cells contribute to the tumour microenvironment (Whiteside, 2008). Various features of the tumour microenvironment result in favourable conditions for the initiation, proliferation, growth, angiogenesis and metastasis of tumour cells (Weber and Kuo, 2012).

It was initially considered that cells in the microenvironment were present to provide immune surveillance and remove tumour cells (Joyce and Pollard, 2009); however recent evidence suggest that infiltrating immune cells provide favourable environmental conditions for the progression of cancers and may suppress the host's immune

response, facilitating metastasis (Whiteside, 2008). Albini and Sporn highlighted some of the targetable features of the tumour microenvironment using chemoprevention including COX 1/2, hypoxia-inducible factor 1α (HIF-1α), TGFβ and VEGF (Albini and Sporn, 2007). There has also been a drive towards re-programming of stromal cells in the tumour microenvironment so that they have an anti-tumourigenic effect (Quail and Joyce, 2013).

1.4.7 The tumour microenvironment in HCC

In HCC, the tumour microenvironment is emerging as a current topic of interest. In one study, assessment of tumour resections for the presence of tumour infiltrating lymphocytes (TILs) showed that increased numbers of TILs and increased expression of inflammatory and immune genes led to increased patient survival (Chew et al., 2010). It was also noted that numbers of NK and T cells were increased in HCC resections compared to non-tumour tissue and this correlated positively with the number of apoptotic cells and negatively with the number of proliferating tumour cells (Chew et al., 2010). In general, the tumour microenvironment in HCC is associated with increased production of cytokines e.g. IL6; chemokines e.g. chemokine (C-X-C motif) ligand (CXCL2), chemokine C-C motif ligand 20 (CCL20); fibrosis; matrix metalloproteinases (MMPs) which can degrade the basement membrane; reduce pO2 resulting in hypoxia; and increase production of ROS resulting in oxidative stress (Hernandez-Gea et al., 2013). There is also a change in the number of cell types that contribute to the tumour microenvironment including: increased numbers of CD4+ Tregs, tumour-associated fibroblasts (TAFs), tumour-associated macrophages (TAMs) and a decrease in the number of dendritic cells leading to a reduced ability to produce an immune response against tumour cells (Hernandez-Gea et al., 2013). Adipocytes have recently been recognised as cells that contribute to the tumour microenvironment which is relevant to patients with NAFLD (Albini and Sporn, 2007). Targets associated with the tumour microenvironment in HCC include STAT3 which is activated via the cytokine interleukin-22 (IL22) secreted by T-helper 17 (Th17) cells and Kupffer cells and is associated with a poorer prognosis (Hernandez-Gea et al., 2013, Jiang et al., 2011).

1.4.8 Neutrophils in HCC

Neutrophils are the most abundant leucocyte comprising 50-70% of circulating WBCs (Sionov et al., 2015). Inappropriate activation of neutrophils induces liver damage alluding to the conflicting role of neutrophils in HCC (Xu et al., 2014). In cancer, neutrophils are attracted to the tumour environment through TAMs and tumour cells (Mantovani et al., 2011). Tumour associated neutrophils (TANs) exhibit opposing roles in cancer dependent on their phenotype (N1 or N2) which is determined by the presence of environmental signalling molecules such as TGFβ (Fridlender et al., 2009, Piccard et al., 2012). The role of N2 neutrophils include: tumour growth, invasion, metastasis, angiogenesis (increased MMP9 and VEGF production), the evasion of apoptosis, genetic instability and promotion of EMT (Dumitru et al., 2013, Piccard et al., 2012, Sionov et al., 2015, Imai et al., 2005). Conversely, neutrophils with an N1 phenotype have a cytotoxic role in response to tumour cells (Xu et al., 2014). In HCC, neutrophils have been recognised as key immune cells in the inflammatory response associated with various aetiologies of HCC (Xu et al., 2014). Neutrophil depletion in DEN murine models resulted in attenuation of liver tumours, indicating that neutrophil inhibition in CLD may be a therapeutic avenue for HCC (Wilson et al., 2015). NFκB was shown to exert a tumour suppressor effect with NFκB p50:p50 dimers able to repress transcription of a neutrophil chemokine network including calprotectin, CXCL1 and CXCL2 (Wilson et al., 2015). Multiple studies have shown that it is the ratio of neutrophils to lymphocytes that has predictive prognostic value in HCC and this is further discussed in **Section 1.4.12**.

1.4.9 Tumour infiltrating lymphocytes in HCC

In cancer, the presence of infiltrating lymphocytes has been recognised as a good prognostic indicator, with attempts to use this as immunotherapy dating back to 1986 (Rosenberg et al., 1986). More recent studies have explored the effects of different subtypes of T-lymphocytes and their ratios in attempt to elucidate whether or not a specific phenotype of T-lymphocytes has a more favourable effect on prognosis. A meta-analysis was performed across various cancer types revealed that in general CD3+ and

CD8+ lymphocytes were positively associated with increased survival benefit; the presence of CD4+ TILs had a less pronounced effect on survival and Forkhead box P3 (FoxP3) TILs were not associated to an improvement in survival (Gooden et al., 2011). The same study concluded that differences may be observed for different cancer types and that in some cases, the ratios of subsets of TILs might be more indicative in terms of prognosis (Gooden et al., 2011).

The role of B-lymphocytes in cancer is less well established. Recent research has alluded to the effect of CD20+ B-lymphocytes and the association with T-lymphocytes as having a positive outcome on survival in cancer (Nelson, 2010). NK cells were identified as possessing cytotoxic properties towards cancer cells (Herberman et al., 1975). It has been considered that the mechanism of action by which NK cells exert cytotoxic properties towards tumour cells may be reliant on the recognition of cells to recognise autologous changes surface markers (Waldhauer and Steinle, 2008). Follow-up studies over 11 years demonstrated that decreased counts of cytotoxic NK cells in peripheral blood led to an increased risk of developing cancer (Imai et al., 2000).

1.4.10 Activated platelets are involved in the immune response and are involved in the growth and metastasis of tumours

The role of platelets in cancer was recognised by Trousseau in the late 19th century; who associated an increase in thrombotic events in patients with cancer (Stanger and Kahn, 2013). This led to the understanding of the role of platelets in cancer including the part they play in the process of metastasis in addition to tumour progression, angiogenesis, cell invasion, vascular permeability, mitogenesis and the production of pro-inflammatory cytokines and chemokines (Gay and Felding-Habermann, 2011, Smyth et al., 2009). Increased circulating platelet counts – or thrombocytosis - has been associated with various cancer types, including ovarian and breast cancer (Rao and Rao, 2012). It has also been proposed that platelets serve as protective cells that are able to assist CTCs evading the immune system (Plaks et al., 2013).

In a HBV transgenic murine model of HCC associated with chronic inflammation, administration of aspirin and clopidogrel alone or in combination resulted in a reduction of HBV specific (CD8+ T-cells) and non-specific inflammatory cells (neutrophils, macrophages, dendritic cells and NK cells) proposing platelets could be a potential therapeutic option in HCC patients (Sitia et al., 2012). In one cohort of 634 HCC patients, 52/634 (8.2%) patients were identified as having above normal platelets (>400 x 10^9/L) (Carr and Guerra, 2013). In this study, elevated platelets were associated with an increased tumour size compared to patients with a platelet count within the normal reference range (Carr and Guerra, 2013).

1.4.11 Tumour-associated macrophages (TAMs)

The population of macrophages in the liver is described as heterogeneous with varying functions ranging from pathogen clearance to promoting liver fibrosis (Tacke and Zimmermann). Hepatic macrophages that reside in the lumen of the liver sinusoids - Kupffer cells - are capable of releasing multiple inflammatory mediators including cytokines and reactive oxygen species (ROS) (Tacke and Zimmermann). Two overarching types of macrophages have been described: pro-inflammatory (M1) or immunoregulatory (M2), though these two classifications of macrophages are further sub-divided dependent on their biological properties (Murray and Wynn, 2011).

In addition to Kupffer cells, there are TAMs that have been recognised as having multiple pro-tumourigenic roles including: angiogenesis, proliferation, metastasis, re-modelling of the ECM and suppression of the host immune response (Weber and Kuo, 2012, Murray and Wynn, 2011). Macrophages with an M2 phenotype have been implicated to be pro-tumorigenic in HCC in both *in vitro* and *in vivo* studies (Yeung et al., 2015). Assessment of 95 HCC samples using IHC and quantitative PCR (qPCR) demonstrated that there was an increase in macrophage and M2 macrophage markers (CD14, CD68 and CD163) and that that this increase in expression was more pronounced in the peri-tumoural tissues compared to the intra-tumoural tissues (Yeung et al., 2015). In cell line studies, co-culturing MHCC97L cells with macrophages led to an increase in number of cells and

migration when M2 macrophages were used but not M1 (Yeung et al., 2015). In HCC, there is activation of stellate cells and TAMs which lead to a release epidermal growth factor (EGF), chemokines, MMP and VEGF (Hernandez-Gea et al., 2013, Wu et al., 2012).

1.4.12 Circulating immune cells

It has been considered that an increase in the counts of immune cells is indicative of immune response in patients, but additionally it may be the ratio of subtypes of immune cells that may be more reflective of the immune status of individuals. Commonly, the neutrophil to lymphocyte ratio (NLR) has been calculated for patients with HCC and has been shown to serve as a useful prognostic indicator in patients receiving a variety of treatment regimens. A higher NLR has been associated with a worse prognosis in terms of PFS and OS in multiple studies comparing cohorts of HCC patients receiving different treatments. In a study of patients with HCC in China, an immune-inflammation score was developed using the neutrophil: lymphocyte ratio as well as the platelet count (Hu et al., 2014). Patients with a high systemic immune-inflammation index (SII) had a shorter OS and relapse-free survival compared to patients with a low SII; furthermore SII correlated with large tumours, vascular invasion and CTC count using the CellSearch (Hu et al., 2014).

Although evaluation of the tumour microenvironment may be more indicative of the factors that are specifically driving the tumour, obtaining this information is not practical in the majority of patients. If clinically relevant information could be obtained from blood sampling, this would be ideal. Assessment of patient circulating immune system status - in terms of numbers and phenotypes of immune cells or through microarray studies to identify differentially expressed genes in peripheral blood mononucleocytes (PBMCs) - may yet yield clinically relevant information that will aid in the future management of patients.

Chapter 2. Imagestream method development and optimisation

In order to detect CTCs in HCC patient samples, it was first important to develop a robust method of CTC isolation and detection. A major difficulty in detecting CTCs is their rarity (≤1 CTC/ml or ≤1 CTC/billion haematopoietic cells) amongst a high background of RBCs, WBCs and platelets (Allard et al., 2004, Yu et al., 2011). Due to the heterogeneity of HCC, CTCs may vary in size and biomarker expression. Furthermore, the expression of a single epithelial marker may not be consistent during the process of metastasis – it has been suggested that CTCs may undergo EMT as part of the process of metastasis (Yilmaz and Christofori, 2009). Difficulty in deciding the most suitable method stems from there having been little characterisation of CTCs in HCC. It has been hypothesised that cancer cells may have increased deformability which aids in the process of metastasis, enabling CTCs to travel through restricted spaces (Byun et al., 2013).

2.1.1 CTC Isolation

Most downstream CTC applications require an enrichment technique to reduce the background of haematopoietic cells and make the possibility of detecting CTCs more likely. Enrichment techniques can be reliant on the physical properties of CTCs for example the ISET method which relies on size of CTCs (Vona et al., 2000); the DEParray which isolates CTCs using dielectrophoresis (Peeters et al., 2013); or density-gradient centrifugation methods where it is hypothesised that CTCs will be captured within the PBMC layer (Rosenberg et al., 2002). Alternatively, enrichment can be performed using the epitope characteristics of cells. Positive enrichment can be used by implementing a tumour cell-specific marker to select positively expressing cells, for example using an EpCAM-coated chip (Nagrath et al., 2007). Negative enrichment can be used to deplete the sample of WBCs using the common leucocyte antigen CD45 (Alix-Panabieres and Pantel, 2014).

2.1.2 CTC detection platforms

The FDA-approved CellSearch system (Janssen Diagnostics, 2016) remains to be the only clinically validated CTC detection platform in metastatic breast, prostate and colorectal cancer (Cohen et al., 2008, Cristofanilli et al., 2004, de Bono et al., 2008). Studies that have been compiled using the CellSearch system have determined a threshold number of CTCs that can be used to predict prognosis in terms of overall survival (OS) and PFS. This is variable amongst the different cancer types for which the CellSearch system has been clinically validated with cut-off levels of ≥5 CTCs in metastatic breast and prostate cancer but ≥3 in metastatic colorectal cancer (Cohen et al., 2008, Cristofanilli et al., 2004, de Bono et al., 2008). Studies implementing the CellSearch system have quoted variable recovery rates using cell spiking experiments ranging from 4.3%-14% (Ghazani et al., 2012) to 80-82% (Riethdorf et al., 2007).

A CellSearch study in a small cohort of pre-treatment HCC patients (n=20), CTCs were detected in 7 patients (45%) (Zee et al., 2007). In a study by Wege and colleagues, CTCs were detected in 18/59 (30.5%) of HCC patients using the CellSearch and these 18 CTC-positive patients had a shorted median OS compared to those that were CTC-negative (460 days vs 746 days) (Schulze et al., 2013). Another research group enumerated CTCs in 50 HCC patients using the CellSearch system finding between 1-8 CTCs per 7.5 ml in 14/50 samples (28%) (Morris et al., 2014). In this study, 19 samples were also assessed using the ISET method where results showed that CTCs were detected in all of these 19 samples of which all had GPC3-positive CTCs (Morris et al., 2014).

2.1.3 The Amnis Imagestream as a CTC detection platform

Despite technological advances there are still many advantages and disadvantages to each CTC detection platform. An ideal CTC detection platform would be sensitive due to the rarity of CTCs. It should also be specific and produce high-resolution images – to ensure that the cells detected are cancer cells. In order to be able to implement it

successfully clinically, it should be fast so that results can be processed in a reasonable amount of time.

The Imagestream (Amnis, Seattle) combines the quantitative capabilities of fluorescent-activated cell sorting (FACS) with the qualitative results of multi-channel, high-resolution and high-throughput fluorescent microscopy. Up to ten fluorescent channels are available for the user to implement biomarkers of interest and high resolution bright field images are produced alongside. Analysis is performed using IDEAS® software (Amnis, Seattle) which enables the user to interrogate multiple cell features and also enable the visualisation of each event recorded. Limited work has been published on the use of the Imagestream as a CTC detection platform. One study comparing CellSearch and Imagestream spiked varying numbers of PANC-1 cells into 7.5 ml blood samples and processed them using the CellSearch or subjected them to an EpCAM-positive enrichment followed by detection using the Imagestream (Lopez-Riquelme et al., 2013). The study concluded that there was no significant difference between the two methods in terms of recovery rates although the Imagestream may be less accurate when low numbers of cells were spiked; however it is important to note that the two technologies were not directly comparable since different enrichment methods were used (Lopez-Riquelme et al., 2013). The perceived advantages and disadvantages of the CellSearch and Imagestream have been summarised in **Table 2.1**. To date there have been no comprehensive studies of using the Imagestream as a CTC detection platform in cancer patients.

Optimal features	CellSearch	Imagestream
Independent of epithelial biomarkers	Dependent on EpCAM and CK	User may select antibodies of choice
Characterisation of cells	CTCs defined as EpCAM+ve/CK+ve/CD45-ve and nucleated	Interrogation of up to 10 cancer-specific biomarkers
Sensitive	Able to detect ≥1 CTC/7.5 ml of blood	Able to detect ≥1 CTC/8 ml of blood
Specific	Baseline levels of CTCs defined for different types of cancer. Biomarkers identify cells of epithelial origin	Able to implement cancer-specific biomarkers
Fast	Processing time ~24 h	Processing time ~4 h with enrichment
Clinically validated	FDA-approved	Not yet clinically validated
High resolution images	Magnification x20 available	Magnification x60 available

Table 2.1 Comparison of the CellSearch and Imagestream CTC detection platforms.

2.1.4 CTC Biomarkers

Initially, in the method development phase, it was decided that classical CTC epithelial biomarkers EpCAM and CK should be included in the biomarker panel since previous studies have shown that these two markers can be detected in HCC CTCs (Morris et al., 2014, Schulze et al., 2013). Furthermore, EpCAM-positive CTCs have been associated with increased tumour-initiating potential (Sun et al., 2013) and serum CK-18 which is detected by the pan-CK antibody has been shown to be an independent predictor of NASH (Feldstein et al., 2009). Since AFP is the only currently used clinical serum biomarker, it was also included. GPC3 was also included due to its overexpression in HCC and the possibility of it being used as a therapeutic target (Filmus and Capurro, 2013). In a study carried out by the Dive group, GPC3 expression was observed in CTCs isolated using the ISET followed by downstream IHC (Morris et al., 2014). CTC GPC3 expression correlated with GPC3 expression in matched patient tumour biopsies although this was in a small patient cohort (n=5) (Morris et al., 2014).

Two additional exploratory markers – DNA-PK and SULF2 – were also included in the study **Sections 1.3.6 and 1.3.5**. SULF2 desulfates GPC3 enabling it to act as a morphogen promoting Wnt signalling (Lai et al., 2008). It is hypothesised that if SULF2

expression could be detected in CTCs, it could be used as a biomarker for stratification with anti-GPC3 or anti-SULF2 treatments. Previous data has shown that DNA-PK expression in HCC patient biopsies is a predictor of patient response to TACE indicating that if it was possible to detect DNA-PK expression in CTCs from the peripheral blood it could serve as a less invasive test to predict patient response (Cornell et al., 2015).

2.2 Aims

- To develop a suitable CTC enrichment technique that is possible to implement for use with the Imagestream.
- To test the recovery rate of cells using an enrichment method in combination with the Imagestream using spiking experiments.
- To develop an analysis strategy using the IDEAS® software to identify CTCs amongst a background of haematopoietic cells.
- To develop an antibody panel for the detection of CTCs in HCC.

2.3 Materials and methods

2.3.1 Mammalian cell culture

HCC cell lines HepG2, Hep3B, Huh-7, PLC/PRF/5, SNU182 and SNU475 acquired from ATCC and ECACC were grown in RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (FBS) (Gibco, UK) or Dulbecco's modified eagle's medium (DMEM) with 15 mM HEPEs, pyridoxine and NaHCO3 (Sigma-Alrich, UK) supplemented with 10% FBS and 2.5% 200 mM L-Glutamine solution (Sigma-Alrich, UK). PreB697 cells were used as a positive control for CD45 and were kindly donated by Dr Lindsay Nicholson. SJSA1 osteosarcoma cells were kindly donated by Dr Arman Esfandiari. Cells were sub-cultured when 70-80% confluent by removing the media, washing with PBS and adding an appropriate volume of trypsin EDTA then incubating for 2-5 min until cells detached. Following cell detachment, trypsin was neutralised with an appropriate volume of media and cells were spun at 1000 x g for 5 min. The supernatant was removed and the cell pellet was re-suspended in an appropriate volume of fresh media. Cells were seeded back down at an appropriate density depending on the cell line or cells were counted using an improved Neubauer haemocytometer (Hawksley, UK) for further application.

2.3.2 Ficoll-Paque™ density gradient centrifugation

2 ml blood samples collected from healthy volunteers in EDTA tubes were spiked with a 1 ml suspension of 5000 Hep3B cells. Samples were diluted in 4 ml of AutoMACS rinsing solution (Miltenyi Biotec) pH 7.2, 2mM EDTA. Diluted blood samples were carefully layers over 4.5 ml of Ficoll-Paque™ PLUS (GE Healthcare) in a 15 ml Falcon tube. Samples were centrifuged at 400 x g for 40 min at 20 ^{o}C. This resulted in separation of the blood sample into plasma, PBMCs, density gradient medium and RBCs in order from top to bottom based on density **Figure 2.1**. The top plasma layer was carefully removed. The following PBMC layer was transferred to a clean 15 ml Falcon tube. AutoMACS rinsing solution was added at a volume of at least three times the volume of the PBMC

layer. Cells were spun at 100 x g for 10 min at 20 °C. The supernantant was gently removed to prevent disturbance of the cell pellet. 6 ml of fresh AutoMACS rinsing solution was added and cells were spun at 100 x g for a further 10 min at 20 °C. The supernatant was removed and the cell pellet was ready for immunofluorescent antibody staining.

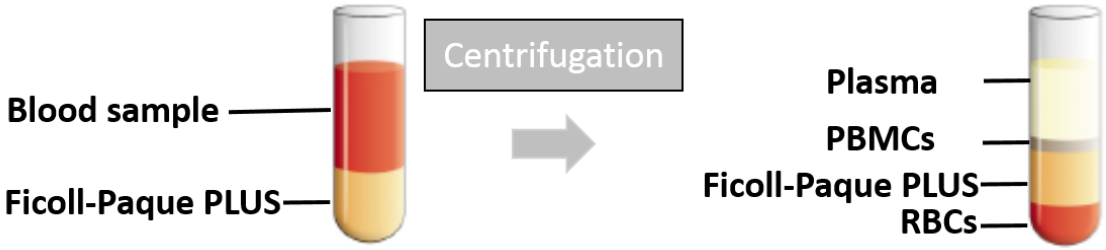


Figure 2.1 Separation of blood sample into plasma, PBMCs and erythrocytes. Adapted from GE healthcare.

2.3.3 Density gradient centrifugation using OncoQuick™ tubes

OncoQuick™ tubes (Greiner Bio-one) contain a porous barrier on top of a density gradient medium. Rather than isolate CTCs with the PBMC layer, the density gradient medium in OncoQuick™ tubes claims to eliminate granulocytes, lymphocyte and mononucleocytes as well as RBCs. A wash buffer was prepared by making up a PBS (Gibco) solution containing 0.5% BSA (Sigma). The centrifuge and OncoQuick™ tubes were pre-cooled to 4 °C. 15 ml blood samples were collected in EDTA tubes from healthy volunteers and spiked with 5000 Huh-7 cells in a 1 ml volume. Blood samples were incubated on ice for 15 min. Blood samples were added to OncoQuick™ tubes by gently pipetting the sample down the side of the tube. Samples were centrifuged at 1600 x g for 20 min at 4 °C without a brake. Following centrifugation the samples above the porous layer was divided into a top brown platelet layer above a blue interphase layer that should capture the spiked cells **Figure 2.2**. The platelet layer was carefully discarded leaving 2-3 ml to prevent disturbance of the blue interphase layer. The

interphase layer was then transferred into a clean Falcon tube. The OncoQuick™ tube was rinsed with 5 ml of wash buffer to remove any residual cells. This was added to the interphase layer and the sample volume was made up to a total of 50 ml with wash buffer. Tubes were inverted 5 times and centrifuged at 200 x g for 5 min. The supernatant was removed leaving ~5 ml to prevent disturbance of the cell pellet. Wash buffer was added again to make up the volume to 50 ml. The sample was centrifuged once more at 200 x g for 5 min. The supernatant was removed leaving the cell pellet remaining for downstream antibody staining and Imagestream analysis.

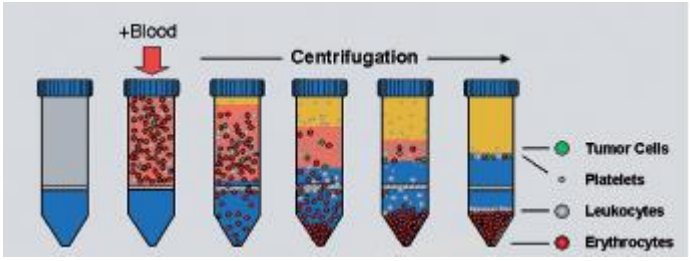


Figure 2.2 OncoQuick CTC enrichment tubes contain a porous filter above a density gradient medium. Following centrifugation, CTCs and platelets are retained above the porous filter whereas leucocytes and erythrocytes pass through the filter. Adapted from Greiner Bio-one.

2.3.4 RBC lysis

8 ml of 5x phosflow lyse/fix buffer (BD Bioscience, US) was diluted in 32 ml of diH2O and pre-warmed to 37 ^{o}C. 50 ml Falcon tubes were blocked with BSA solution (MACS) diluted 1:20 in AutoMACS wash solution. 5 ml of the resulting solution was used to block each Falcon tube. Following blood collection, the BSA solution was removed from the tube and 8 ml of the blood was transferred to the blocked Falcon tube. This was stored at 4 ^{o}C for 15 min. 200 ml of FcR blocking reagent (MACS) was added per 8 ml of blood and incubated for 15 min. The sample was then divided up into 2 ml aliquots in blocked Falcon tubes. Following 30 min incubation at 37 ^{o}C, 40 ml of the 1x phosflow lyse/fix buffer was added to each 2 ml of aliquot of blood. The tube was inverted 10x and then

incubated at 37 ^{o}C for 10 min. The RBC lysis was then centrifuged at 500 x g for 8 min at RT. The supernatant was discarded and the remaining cell pellet was re-suspended in PBS and transferred to a siliconised eppendorf tube (Sigma Aldridge, UK). The Falcon tube was washed 4 times to retrieve any remaining cells and then the cells were spun at 470 x g for 5 min at RT. The supernatant was removed and the cell pellet re-suspended in 1 ml of permeabilisation/wash buffer containing saponin (BD Biosciences) for 1 h at RT. Following incubation, the cell suspension was centrifuged at 470 x g for 5 min and the remaining cell pellet was re-suspended in 100 µl of permeabilisation/wash buffer to which antibodies were added. Following antibody incubation, cells were spun at 470 x g for 5 min and the pellet was re- suspended in an appropriate volume of PBS. Samples were run on the Imagestream (Amins, US).

2.3.5 Immunomagnetic CD45 depletion

The above protocol **Section 2.3.4** was followed with the additional step of a negative enrichment step - CD45 depletion using immunomagnetic CD45 coated nanoparticles **Figure 2.3**. Following the RBC lysis, the supernatant was removed and the remaining cell pellet was re-suspended in 500 µl of RoboSep buffer (Stem Cell technologies). The suspension was transferred to a 14 ml polystyrene round-bottom tube (BD Falcon) and the previous Falcon tube was washed with 4 x 1 ml volumes of 10% PBS/BSA solution to remove any residual cells. Cells were spun at 500 x g for 8 min and the remaining cell pellet was re- suspended in 500 µl of RoboSep buffer. EasySep™ CD45 depletion cocktail was added for 15 min followed by 50 µl of EasySep™ magnetic particles for 10 min incubation. The volume of cell suspension was made up to 5 ml with RoboSep buffer and the tube was then incubated in the magnet for 10 min. The unbound cells (CD45 negative) were poured off into a blocked falcon tube and a subsequent wash of the magnet was carried out. Cell suspensions were centrifuged for 5 min at 800 x g and then permeabilised using either ice cold methanol for 20 min or Perm/Wash™ (BD Biosceinces) buffer containing saponin for 1 h at RT. Following permeabilisation, cells were spun at 250 x g for 5 min and re-suspended in 100 µl of Perm/Wash™ buffer or 1% BSA/PBS solution. Antibodies **Table 2.2** were added in the order described in **Table 2.3**.

Following antibody incubation, cells were spun at 470 x g for 5 min and re-suspended in an appropriate volume of PBS, then processed through the Imagestream.

Primary/conjugated antibodies	Species	Dilution	Company (catalogue #)
488 anti-human CD326 (EpCAM) antibody (clone 9C4)	Mouse	1:50	AlexaFluor (324210)
PerCP/Cy5.5 CD326 (EpCAM) antibody (clone 9C4)	Mouse	1:50	Biolegend (324214)
Cytokeratin PE (C-11)	Mouse	1:100	Cayman Chemical (10478)
V450 anti-human CD45 (clone HI30)	Mouse	1:20	BD Bioscience (560367)
PE/Cy7 anti-human CD45 (clone HI30)	Mouse	1:50	BioLegend (304016)
Alphafetoprotein (3H8) Mouse mAb (AlexaFluor 594 conjugate)	Mouse	1:20	Cell signalling Technology (7877S)
Glypican-3 (IG12)	Mouse	1:50	Santa Cruz (Sc-65443)
DNA-PKcs (H163)	Rabbit	1:100	Santa Cruz (Sc-9051)
SULF2	Mouse	1:100	Serotec (MCA5692GA)
Secondary antibodies			
Goat anti-rabbit AF488	Goat	1:1000	Invitrogen (A11034)
Goat anti-mouse AF488	Goat	1:1000	Invitrogen (A11001)
DNA dyes			
DRAQ5		1:5000	Cell Signalling Technology (1-877-616-23537)
DAPI		1:2000	Biolegend (422801)

Table 2.2 Antibodies used in the development of an antibody panel for HCC patient samples using the Imagestream.

Order	Antibody/dye	Incubation time
1	Primary un-conjugated	1h
2	Secondary	1h
3	Intracellular	30 min
4	Membrane/DNA dye	1h

Table 2.3 Order of antibody staining for cells processed using the Imagestream.

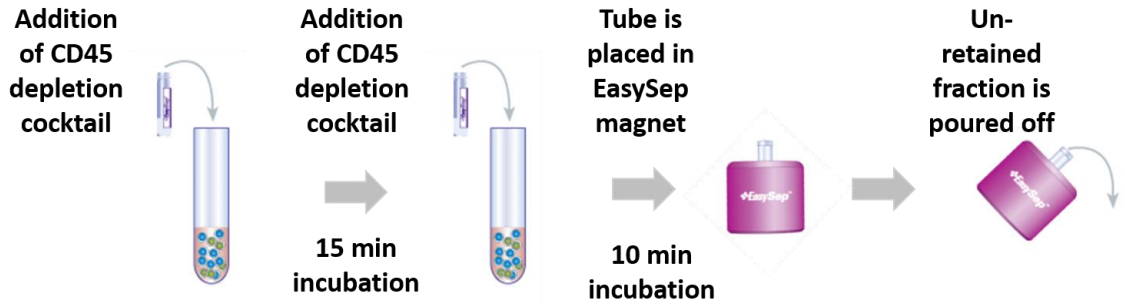


Figure 2.3 Overview of the EasySep™ immunomagnetic depletion. Adapted from StemCell Technologies.

2.3.6 Haematopoietic cell depletion - spiking experiments

A stock suspension of 20,000 Huh-7 cells/ml was made up determined by cell counts using a haemocytometer. From this, serial dilutions were made to produce stock solutions of the lower cell counts so that 2000, 200 and 20 cells were spiked in to 4 ml blood samples in a volume of 100 µl. Samples were processed according to **sections 2.3.4 and 2.3.5**. The resulting cells were stained with fluorescent conjugated antibodies **Table 2.2** against EpCAM (AlexaFluor 488, AlexaFluor), pan-CK (PE, Cayman Chemical) and CD45 (V450, BD Bioscience). DRAQ5 (Cell Signalling Technology) was used as a nuclear stain. Cell pellets were re-suspended in 50 µl of PBS and processed through the Imagestream.

2.3.7 Antibody optimisation

Initial antibody optimisation was carried out using HCC cell lines grown on glass coverslips. 1 x 10^5 cells were seeded out per coverslip in a 6-well plate. Cells were left to grow until ~70-80% confluent then fixed in either 1% formalin for 20 min at RT or in methanol at -20 ^{o}C for 20 min. A permeabilisation step for cells fixed with formalin was included by incubating with a saponin-containing Perm/Wash™ buffer. Cells were washed and blocked with 5% BSA solution for 20 min at RT. Coverslips were then

immersed in 1% BSA solution containing primary antibody for 1 h at RT. Cells were then washed three times with PBS for 5 min and incubated in secondary antibody for 1 h at RT in the dark. Following incubation with secondary antibody cells were washed for three times in PBS then for 10 min with diH_2O. A secondary antibody only control was included to determine any background staining caused by the fluorescent antibody. Coverslips were left to dry then fixed onto slides using Vecta mounting media containing DAPI (Vector Laboratories, US). Slides were viewed using the Leica DMR fluorescent microscope (Leica, Germany).

2.3.8 Antibody staining of cells prior to Imagestream processing

Optimal conditions for antibodies were then tested in cell lines using the Imagestream. Fluorophores were selected on the basis of excitation lasers and emission spectra. Where antibodies were unconjugated, secondary antibody only controls were included and any background was removed during analysis. Single colour controls were also included to compensate for spectral overlap between fluorophores during the analysis process.

Cells from **Section 2.3.1** were fixed in 1% formalin for 20 min at RT. Cells were then centrifuged at 500 x g for 5 min, the supernatant was removed and the cell pellet was incubated in Perm/Wash™ buffer for 1 h at RT. A further 5 min centrifugation at 500 x g was used to pellet cells, the supernatant was removed and cells were re-suspended in 100 µl of fresh Perm/Wash™ buffer. Antibodies were added at appropriate dilutions **Table 2.2** for 1 h at RT. Cells were centrifuged again at 500 x g, supernatant removed and replaced with 100 µl of fresh Perm/Wash™ buffer. Secondary antibodies were added for 1 h at RT with a DNA dye and cells were incubated in the dark. Where multiple antibodies were used, they were added in the order according to **Table 2.3**.

2.3.9 Imagestream processing

Final cell pellets were suspended in an appropriate volume of PBS and aliquoted into 60 µl samples in siliconized Eppendorfs so that the object rate remained below 1000 objects/s when running the sample through the Imagestream. Samples were processed through the Imagestream using an 8 µm core at 60 mm/s with 7% SpeedBeads® to enable calibration and hydrodynamic focussing. Lasers 405 nm, 488 nm, 561 nm and 642 nm were used to excite fluorophores. The 785 nm laser was used to collect side scatter profiles or switched off the enable detection of the PE/Cy7 fluorophore in channel 7. Cell images were captured using a 40x objective and charged-coupled device (CCD) camera.

2.3.10 IDEAS® Analysis –measuring biomarker expression

To measure expression of the relevant biomarkers in a panel of HCC cell lines, the mean pixel intensity feature in IDEAS® was used. Firstly, round single cells were identified by creating a scatterplot of aspect ratio against brightfield area. The round single cell population was gated and boundaries were checked to ensure that no double cells or debris was included. This population was then gated for the in focus cell population by creating a histogram of gradient root mean square which measures large differences in pixels, with objects in focus usually having a gradient root mean square value of >50. The focussed, single cell population was then assessed for the mean pixel intensity of the biomarker of interest.

2.3.11 IDEAS® Analysis –gating strategies to identify spiked cells

Data from the Imagestream was initially in the form of a raw information file. A compensation matrix built using the single colour control was applied to this file to compensate for spectral overlap between fluorophores used. Where unconjugated antibodies were used, background from the secondary-only antibody control was removed from the appropriate channel. Scatterplots of EpCAM or CK intensity versus

CD45 intensity were plotted. Populations with a low CD45 intensity and high EpCAM/CK intensity were gated. To exclude the possibility of counting spiked cells twice, a further population of either EpCAM+ve/CD45-ve or CK+ve/CD45-ve was created. This population was then inspected by eye and cells that had a consistent cell morphology based on the brightfield image, were CD45-ve, EpCAM/CK+ve and nucleated were tagged to create a population of spiked cells. This population was then enumerated and recovery was calculated as a percentage of the total cells spiked.

2.4 Results

2.4.1 Erythrocyte depletion of whole blood samples resulted in high CTC recovery but large, unmanageable data files

It is possible to stain whole blood and process it through the Imagestream but in an 8 ml blood sample, this would result in approximately 3.04 - 4.96 x 10^{10} erythrocytes, 1.12 – 3.6 x 10^{9} platelets and 8.8 -28 x 10^{6} leucocytes resulting in data files containing >30 billion images. Even with stringent gating techniques, management and analysis of this data would be unfeasible. To reduce the majority of erythrocytes, samples were subject to a RBC lysis as described in **Section 2.3.4**. Although minimal processing following a RBC lysis led to a high recovery of cells, processing took up to 24 h and data files were unmanageable to continue this method in large numbers of patients.

2.4.2 Density gradient centrifugation methods resulted in a poor recovery of spiked cells

As a collaborative effort between people working on CTCs indifferent cancer types – namely Dr David Jamieson, Mr Barry Dent and Dr Rachel O'Donnell – different enrichment approaches were tested. These included the CD45-coated beads (MACS) and DynaBeads® (Thermo Fisher); MagSweeper (Talasaz et al., 2008) and RosetteSep™ (Stemcell Technologies). Aside from the RosetteSep™, which is a density gradient centrifugation method that also depletes the sample of unwanted cells by binding them to erythrocytes, recovery rates were poor. The RosetteSep™ achieved good recovery however, also produced large amounts of debris; making it unsuitable for use with the Imagestream since each piece of debris would be counted as an object. Density gradient centrifugation using Ficoll-Paque™ PLUS as described in **Section 2.3.2** achieved poor recovery rates of 12% (data not shown). Similar results were achieved using the OncoQuick™ **Section 2.3.3** tubes (10.2%, data not shown). These methods were clean in that debris was minimal, but recovery rates were low. In parallel,

preliminary results were promising with immunomagnetic depletion using the EasySep™ magnet **Section 2.3.5**, so this method was pursued further.

2.4.3 Immunomagnetic CD45 depletion resulted in ~50% recovery of CTCs and a reduction of ≥95% of CD45+ leucocytes

Recovery rates of three different concentrations of HCC cell lines spiked into healthy volunteer blood were determined. Samples were processed using a protocol that would be transferable to patient sample processing. Following RBC lysis **Section 2.3.4**, removal of platelets by a low centrifugation spin (270 x g) and the immunomagnetic depletion of CD45+ WBCs using the EasySep™ technique **Section 2.3.5**, recovery was 65.7% (SEM±8.2), 51.3 (SEM±10.2) and 57.3 (SEM±3.9) for 2000, 200 and 20 spiked cells per ml respectively. Similar recovery rates were achieved in spiking experiments with cell lines from three different cancer types: oesophageal, ovarian and thyroid (Dent et al., 2015). Leucocyte number pre- and post-depletion samples was measured using a haemocytometer. Results showed that in post-depletion samples there was a reduction in WBCs by ≥95% **Figure 2.5**. This would result in decreased processing time using the Imagestream as well as manageable data files and shorter analysis time.

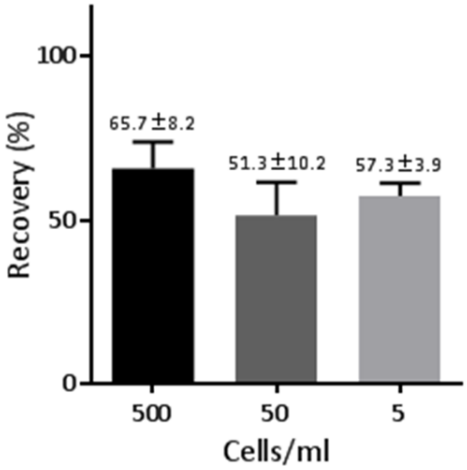


Figure 2.4 Recovery rates for 500 (65.7%±SEM 8.2), 50 (51.3±SEM 10.2) and 5 (57.3±SEM 3.9) Huh-7 cells spiked into whole blood and processed using an erythrocyte lysis and CD45 immunomagnetic depletion.

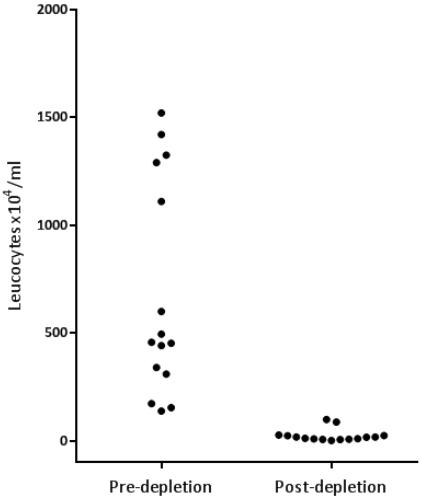


Figure 2.5 Pre and post-depletion counts of leucocytes demonstrated that depletion of CD45 positive leucocytes was consistently ≥95%.

2.4.4 Antibody optimisation of biomarkers of interest for the Imagestream

Antibodies to be included in the biomarker panel were optimised using immunofluorescence in HCC cell lines **Figure 2.6**. For epithelial biomarkers, SJSA1

osteosarcoma cells which have a mesenchymal phenotype were included as a negative control. PreB697 cells were used as a positive control for CD45 and WBCs were used a negative control for other biomarkers of interest. Conditions tested included dilution of primary antibody **Figure 2.7**, fixation and permeabilisation techniques **Figure 2.8** as well as the effects of any preservatives contained in blood collection tubes **Figure 2.9**. Optimum antibody dilutions for antibodies for use with the Imagestream are summarised in **Table 2.2**.

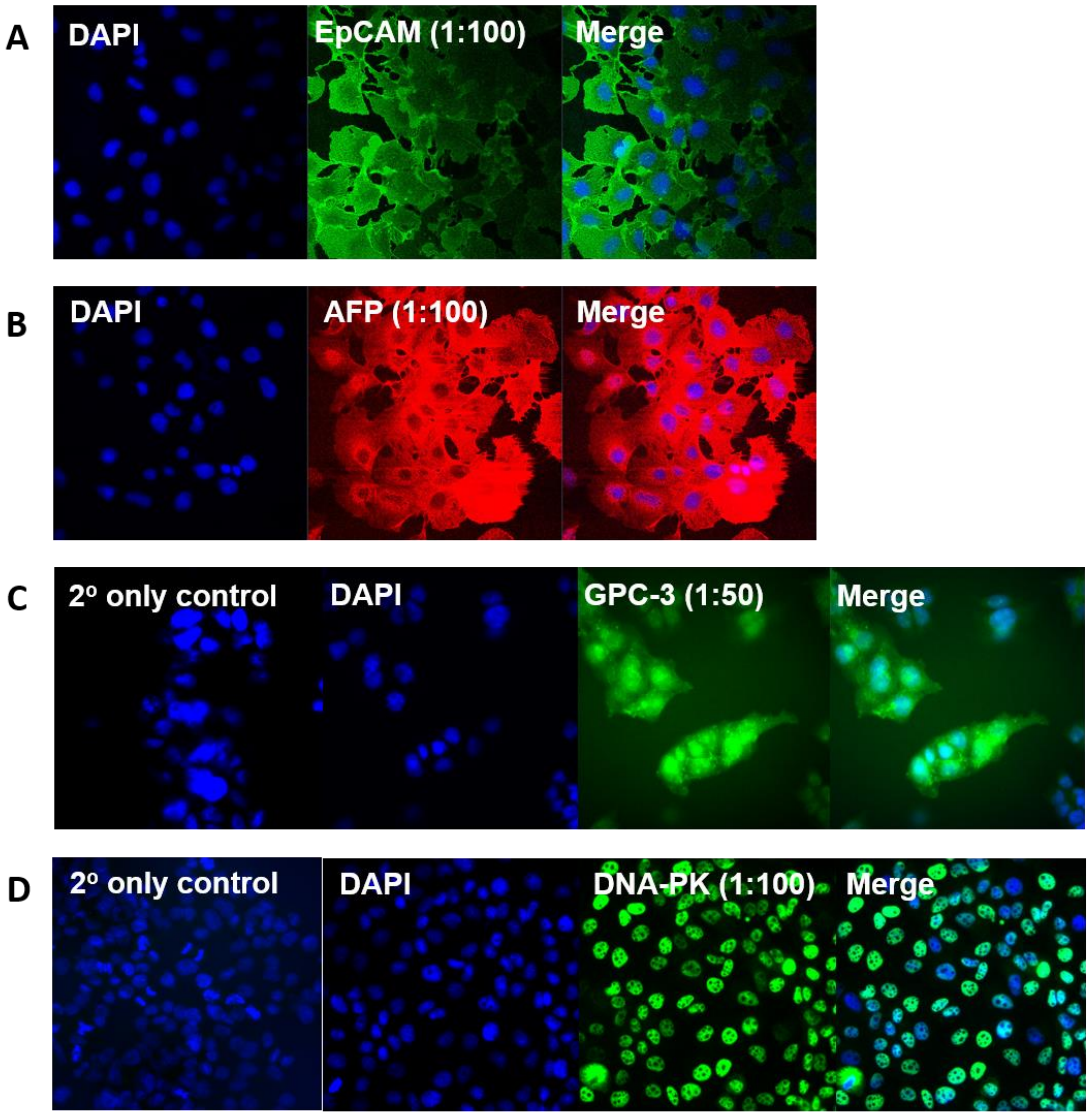


Figure 2.6 Initial optimisation of antibodies for biomarkers of interest was performed using standard immunofluorescence. Secondary antibody only controls were included for unconjugated antibodies (C-D). A. Hep3B cells stained with EpCAM AF488 (AlexaFluor). B.Hep3B cells stained with AFP AF594 (Cell Signalling Technology). C. HepG2 cells incubated with primary glypican-3 (Santa Cruz) and secondary goat anti-mouse AF488 antibody. D. PLC/PRF/5 cells incubated with primary DNA-PK (cs) and secondary goat anti-rabbit AF488 antibody.

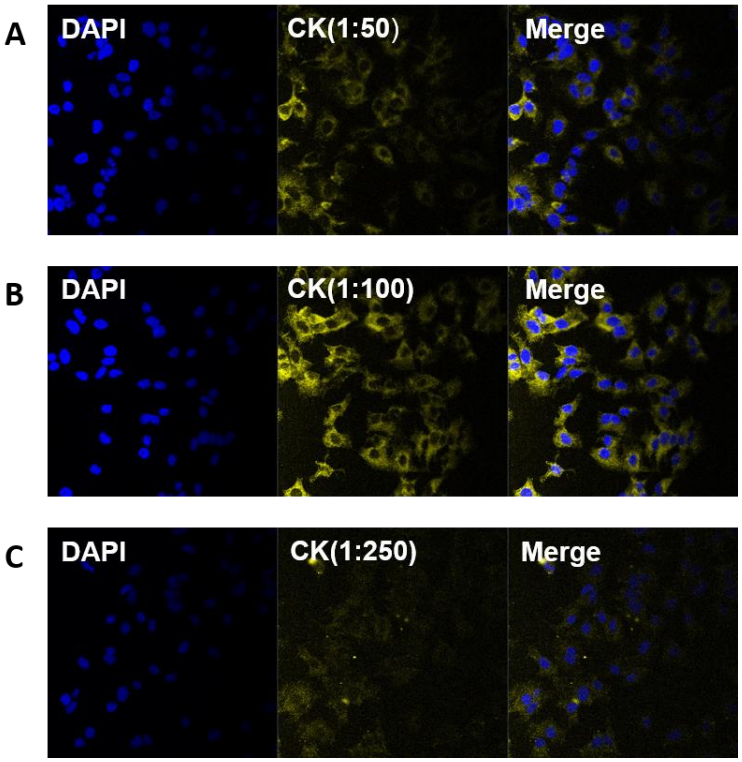


Figure 2.7 Huh-7 cells stained with pan-CK (Cayman chemical) in varying dilutions: A. 1:50, B. 1:100 and C. 1:250.

The RBC lysis buffer contained a fixative (unspecified by the manufacturer) and initially a saponin permeabilisation was used. However, the DNA-PK antibody required an ice cold methanol permeabilisation. In order to determine whether or not this would have any effect on recovery rates, cells fixed in methanol and formalin were processed through the Imagestream and recovery was determined as 96.9% for methanol-fixed cells and 97.9% for formalin fixed cells.

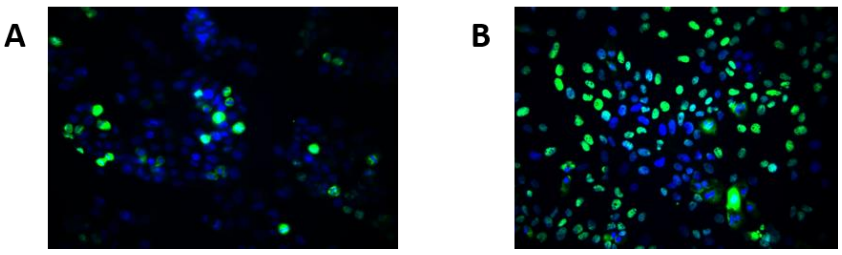


Figure 2.8 Huh-7 cells were fixed with A. formalin followed by a saponin permeabilisation or B methanol.

CellSave tubes are used alongside the CellSearch technology. Data from cell line experiments has shown that these fixative-containing tubes mean that samples can be collected, fixed immediately and can be processed up to 96 h later (Qin et al., 2014). In our HCC cohort, this would mean that it would be possible to collect samples from other cancer centres across the UK and enable sub-cohort analysis of CTCs in patients in specific treatment groups. However, tests using cell lines demonstrated that the fixative (unspecified by the manufacturer) used in the CellSave tubes had an antigen masking effect on DNA-PK **Figure 2.9**. Huh-7 cells fixed and permeabilised using methanol displayed nuclear DNA-PK in 88% of the cell population **Figure 2.9 A**. A magnified image of Huh-7 cells fixed in a CellSave tube displays unspecific DNA-PK staining **Figure 2.9 B**. Only 55% of the cell population was DNA-PK positive indicating antigen masking with the CellSave preservative, despite a methanol permeabilisation. Following this we decided to collect in EDTA tubes and process samples immediately to avoid loss of CTCs.

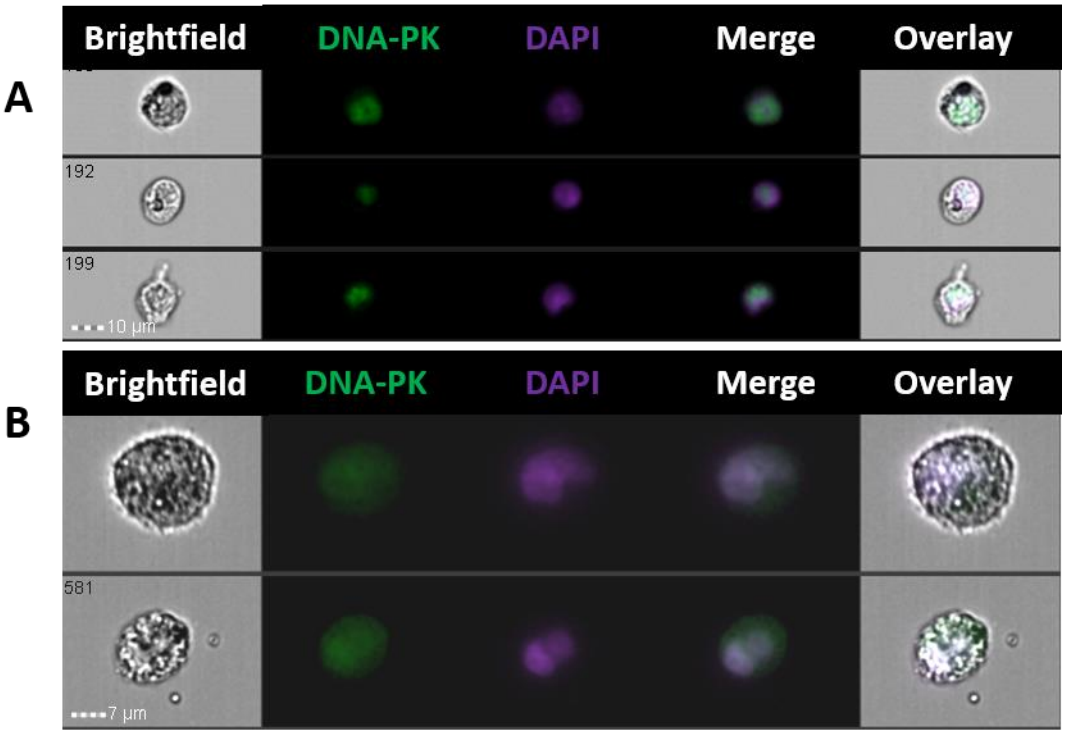


Figure 2.9 Huh-7 cells stained with primary DNA-PKcs antibody (Santa-Cruz) and AF488 (AlexaFluor) goat anti-rabbit antibody. A. Cells were incubated in a K3 EDTA tube for 1h and methanol fixed (x40 objective). The preservative in the CellSave tubes appeared to have an antigen-masking effect in B. Cells fixed in a CellSave preservative tube for 1h followed by a methanol permeabilisation (x60 objective).

2.4.5 HCC cell lines display heterogeneity in biomarker expression

Expression of biomarkers was measured using mean pixel intensity in the IDEAS® software as described in **Section 2.3.10**. Heterogeneity was observed between the HCC cell lines tested and there was variability observed in the range of mean pixel intensities within each cell line **Figure 2.10**. The osteosarcoma cell line SJSA1 which was used as a negative control for the epithelial biomarkers had low expression of EpCAM and CK compared to the HCC cell lines as expected since they have a mesenchymal phenotype **Figure 2.10 A-B**. Hep3B cells had a large range of pixel intensities for EpCAM, AFP and GPC3 but low range for CK compared to the other HCC cell lines **Figure 2.10**. The graph profile of mean pixel intensities of AFP and GPC3 was similar although the range of pixel intensities for GPC3 were generally higher compared to AFP. This data demonstrates the heterogeneity of biomarker expression amongst HCC cell lines,

indicating that the CTC population may also be heterogeneous; supporting the use of multiple biomarkers for the detections of CTCs.

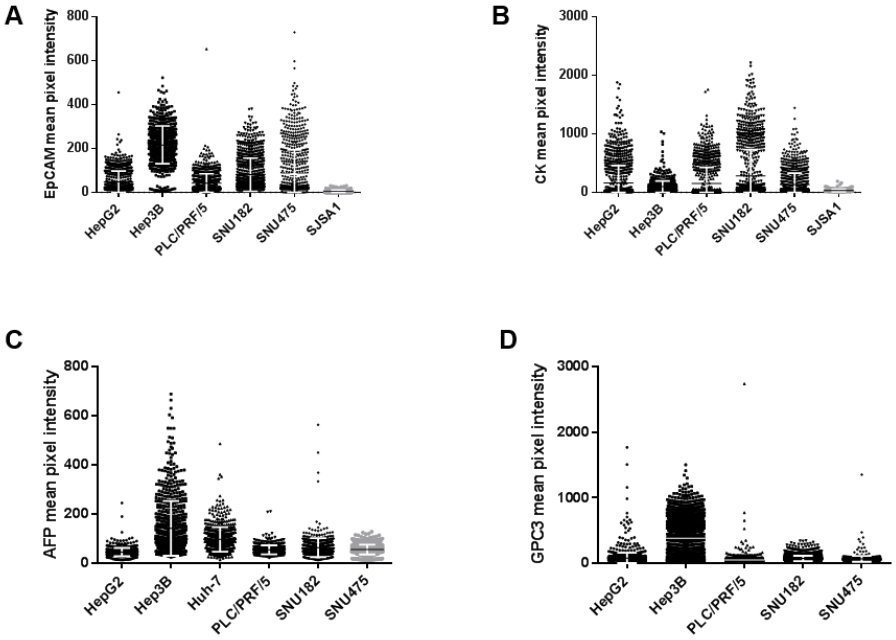


Figure 2.10 Data displaying the mean pixel intensities of A. EpCAM B. CK C. AFP and D. GPC3 in HCC cell lines.

2.4.6 6-channel antibody staining

Although the Imagestream has 12 channels, 2 are retained for brightfield images. Of the remaining 10 channels, different fluorophore combinations were trialled to determine which fluorophores would be best to use for an antigen panel. Initially an antibody panel of EpCAM (AF488), pan-CK (PE) and CD45 (V450) with DRAQ5 as a DNA dye was used **Figure 2.11**. After trialling different antibody combinations, the use of 6 different fluorophores led to a panel that could be applied to detect biomarkers in CTCs in HCC patient samples **Figure 2.12**. It is possible to utilise further fluorophores but there would be increased spectral overlap which would make compensation difficult.

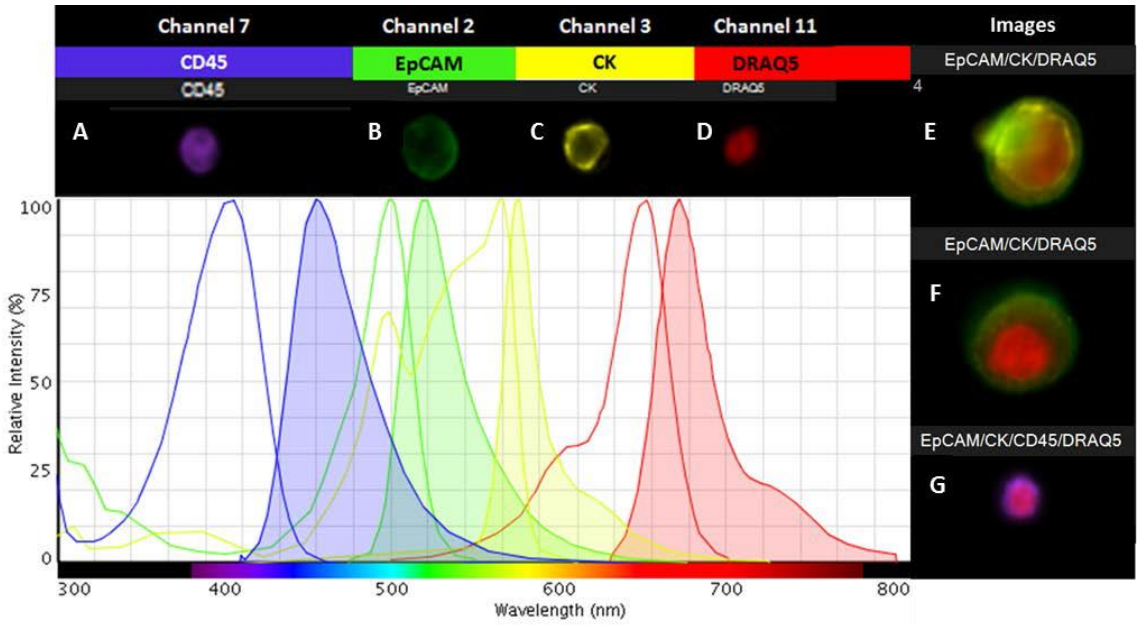


Figure 2.11 PreB697 cells stained with CD45-V450 (BD Bioscience). B. SNU182 cells stained with anti-human EpCAM- AlexaFluor488. C. SNU182 cells stained with pan-CK-PE. D. DRAQ nuclear stain. E-F. SNU182 cells stained with CK-PE and EpCAM-AlexaFluor488. G. CD45 positive WBC stained with all antibodies.

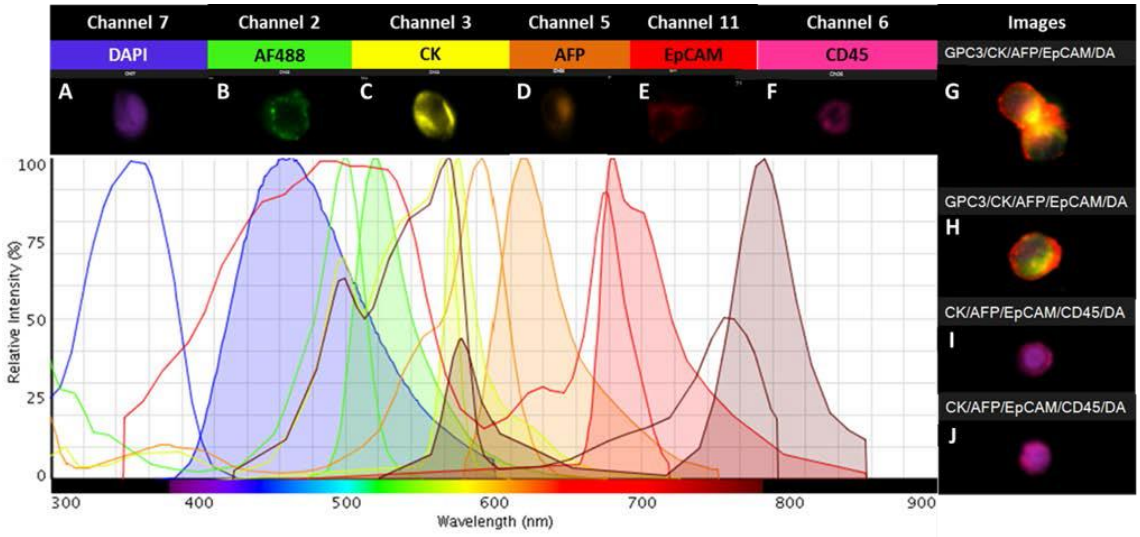


Figure 2.12 Antibody panel that enables detection in six channels. A. Hep3B cells stained with DAPI. B. HepG2 cells stained with glypican-3 (Santa Cruz) primary antibody and goat anti-mouse AlexaFluor488 secondary antibody (Invitrogen). C. Hep3B cells stained with pan-CK-PE (Cayman Chemical). D. Huh-7 cells stained with AFP-AlexaFluor594 (Cell Signalling). E. Hep3B cells stained with EpCAM- PerCP/Cy5.5. F. PreB697 cells stained with CD45-PE/Cy7. G-H. Huh-7 cells stained with all antibodies. I-J. CD45-positive WBCs stained with all antibodies.

2.4.7 HCC CTCs can be gated based on area of the brightfield image

Initially it was not known whether or not CTCs were larger in size compared to haematopoietic cells. Despite depletion, data analysis files produced following compensation still contained large numbers of objects >50 µm in diameter. In order to analyse these data efficiently, a gating strategy was implemented. Based on the CTC definition that objects that were cellular in morphology, nucleated, CD45-negative and positive for one or more of the biomarkers in the panel were classified as CTCs, gating was reliant on biomarker expression.

During analysis, objects that appeared to be cellular in morphology, nucleated, CD45-negative but also negative for biomarkers tested in the CTC panel were observed, indicating that the initial gating strategy may be excluding CTCs that did not express biomarkers in the panel i.e. cells that may have undergone EMT or expressed biomarkers not included in the panel. Additionally, objects that appeared to be larger than cell lines and have a morphology consistent with that of a macrophage were also noted but failed to be detected using the initial gating strategy due to expression of one or more CTC biomarkers and CD45 positivity. A new gating strategy was developed that took into account nuclear intensity. Using a histogram of intensity of the nuclear stain against frequency resulted in distinct populations of cells with a nuclear intensity of single WBCs, double WBCs and anything greater in nuclear intensity than a doublet of WBCs. The single WBC population could then be further gated on the basis of CD45 intensity using a second histogram to identify any CD45 negative objects. Cells with a nuclear intensity greater than a single WBC were then assessed for the expression of candidate biomarkers. A final population of cells that had either a nuclear intensity of a single WBC but were CD45 negative and cells with a nuclear intensity greater than a single WBC and were biomarker positive were combined into a single population and assessed by eye. Objects that had a consistent cellular morphology, were CD45 negative and biomarker positive were tagged to form a population of spiked cells or CTCs.

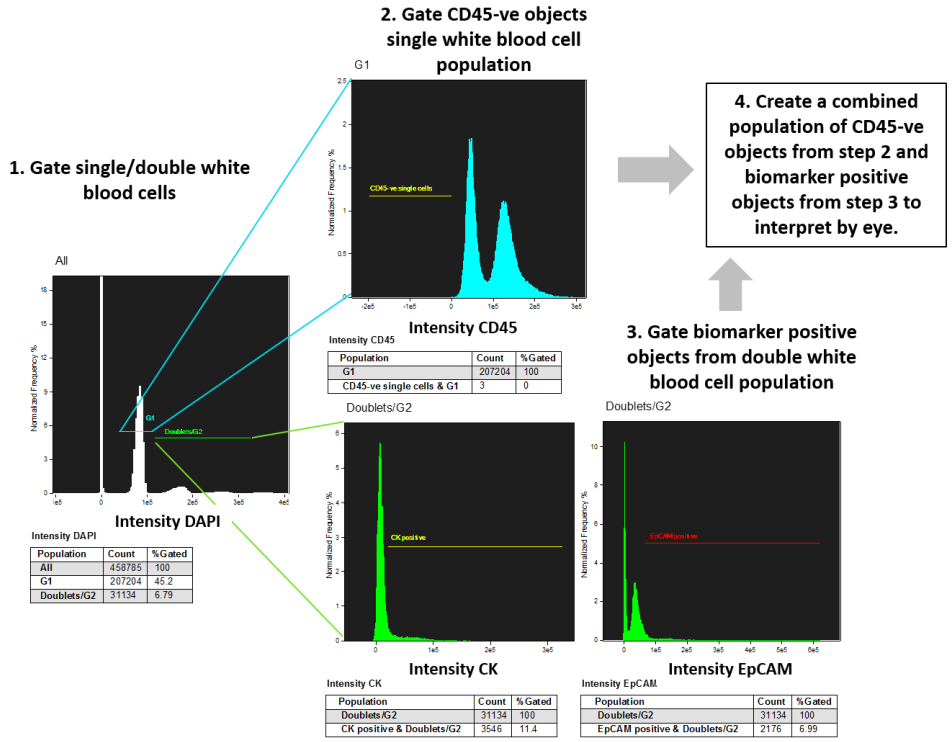


Figure 2.13 Analysis algorithm to identify spiked cells or CTCs against a background of haematopoietic cells.

Following the analysis of blood samples from a HCC patient cohort **(Chapter 3)**, CTCs observed appeared to be larger in size. A mask was modified (standard features brightfield area mask, eroded by 3 pixels) in IDEAS® to measure the area of the brightfield image. This mask was then created as a feature in IDEAS® to measure the brightfield area of objects in a population. When the brightfield area of WBCs, HCC cell lines and CTCs was measured, CTCs had a significantly larger area than WBCs. Of the gating methods tested to identify CTCs in HCC, this last method lead to a reduced final population to be viewed by eye whilst including biomarker negative cells and other objects of interest e.g. macrophages.

2.5 Discussion

Initially, whole blood samples were processed through the Imagestream, though an 8 ml blood sample took ~24 h to run, produced large data files and resulted in a lengthy analysis process. This was not going to be feasible in larger patient cohorts so a pre-enrichment step was required. In HCC there have been no reports in the literature about the size of HCC CTCs, so methods dependent on size e.g. filtration, ISET were not considered. One of the major advantages of the Imagestream is that antibodies of choice can be incorporated into a biomarker panel that is tailored to cancer type. This also means that there is no selection bias towards the CTC population studied. Therefore, enrichment based on expression of epithelial markers was excluded.

Due to lack of knowledge about CTCs in HCC, we hoped to develop a method that would result in the least selection bias in the CTC population detected, did not deplete the sample of any CTCs based on assumptions made on size and that would significantly reduce processing and analysis time compared to using whole blood. Depletion methods tested were mostly based on the depletion of CD45-positive leucocytes. The RosetteSep™, MagSweeper and DynaBeads® were trialled though the former resulted in a large amount of debris and the latter two methods resulted in poor recovery rates. Density gradient centrifugation methods also resulted in poor recovery (~10%). The combination of an erythrocyte lysis along with a CD45 immunomagnetic depletion using the EasySep™ resulted in a recovery of >50% based on cell spiking experiments **Figure 2.4**. It also led to the depletion of ≥95% of CD45 positive leucocytes resulting in a sample that was faster to process using the Imagestream and quicker to analyse using the IDEAS® software **Figure 2.5**.

When generating a biomarker panel for the identification and characterisation of CTCs in HCC patient samples, we decided to include some of the classical epithelial markers that have already been identified on CTCs from HCC patients. These included EpCAM, CK and GPC3 (Morris et al., 2014, Schulze et al., 2013). We also wanted to exploit the potential of the Imagestream by including biomarkers that have not previously been

explored in CTC research. We also wanted to include markers with potential clinical utility in terms of stratifying patients for therapy or those that could be used to predict response (Cornell et al., 2015, Filmus and Capurro, 2013, Lai et al., 2008). Expression of HCC biomarkers was variable amongst HCC cell lines; indicative of heterogeneity which may be observed in the CTC population **Figure 2.10**. The data presented in this chapter show that it was possible to optimise and antibody panel for up to five different fluorescent markers and a nuclear stain **Figure 2.12**. It was decided that EpCAM, CK, AFP, GPC3, DNA-PK and SULF2 expression in CTCs would be explored in HCC patient samples in a series of biomarker panels including CD45 and a nuclear stain.

Initially, a gating algorithm was developed to identify spiked cells or CTCs based on nuclear intensity, the absence of CD45 and the expression of biomarkers **Figure 2.13**. However, despite depletion, this still led to a large population of cells to view by eye. Gating based on measurements of the brightfield area in HCC CTCs following results showing that CTCs were larger than WBCs **Section 3.3.2**, resulted in an efficient gating strategy that also retained the inclusion of other cells of interest e.g. biomarker negative cells and macrophages.

2.6 Conclusion

- Depletion of haematopoietic cells using RBC lysis, a low centrifugation step to deplete platelets and a CD45 immunomagnetic depletion resulted in ≥95% reduction in WBCs with the maintenance of a recovery rate of 51.3-65.37% of CTCs.
- Optimal antibody conditions for biomarkers of interest for use with the Imagestream were determined and panel of 6 different fluorophores was developed for use on patient samples.
- Retrospectively, a gating strategy based on the size of HCC CTCs could be used to improve the speed and efficiency of analysis while maintaining other cells of interest e.g. biomarker-negative cells and macrophages.

Chapter 3. CTCs and ctDNA in HCC patient samples

3.1 Introduction

Patients with HCC often present at an advanced stage when treatment options are limited. Additionally, due to the underlying CLD, standard anti-cancer cytotoxic options for HCC are limited since the potential benefit is often outweighed by the likelihood that such agents will induce further liver damage. Although recently there has been an increase in the number of targeted therapies for use in cancer, there has been little success in HCC; partly owing to the lack of the identification of key molecular drivers of HCC but also due to the lack of identification of biomarkers that could be used in trials for molecular therapies in HCC.

Relatively few studies have investigated the presence of CTCs in patients with HCC (**Section 1.2.8**). Furthermore, studies that have been compiled have mainly focussed on the enumeration of CTCs in patients with HCC rather than the molecular characteristics of CTCs, which is a field of growing interest (Morris et al., 2014, Schulze et al., 2013). It is proposed that characterisation - as opposed to CTC count - may be able to provide clinically relevant information in terms of diagnosis, prognosis, therapy stratification and treatment response or development of resistance.

3.1.1 CTCs in HCC

Studies that have investigated CTCs in HCC have been summarised in **Table 1.4**, Schulze and colleagues tested the CellSearch system for the detection of CTCs in HCC. 18/59 (30.5%) of samples tested positive for CTCs defined as ≥1 CTC/7.5 ml (Schulze et al., 2013). In this study the presence of CTCs correlated with OS, Barcelona Clinic Liver Cancer (BCLC) staging, AFP, macro- and microscopic vessel invasion. It is however important to note that the threshold of ≥1 CTC/7.5 ml is lower than other studies for which the CellSearch is clinically validated and 1/19 of the control patients consisting

of patients with cirrhosis or benign hepatic tumours had a positive result by the definition set by the study itself (Schulze et al., 2013).

Morris and colleagues explored identifying CTCs in HCC using the ISET method and comparing this to use of the CellSearch (Morris et al., 2014). During this study they identified CTCs in 15/50 (28%) of patients using the CellSearch and 19/19 (100%) patients using the ISET method, following which they performed downstream IHC using GPC-3 as a HCC biomarker (Morris et al., 2014). They then went on to correlate GPC-3-positive CTCs with expression of GPC-3 in the primary tumour using tissue biopsy; concluding a correlation of 100% in 5 paired samples (Morris et al., 2014). No associations were made between CTC number and tumour stage or outcome (Morris et al., 2014).

Other investigators have used methods based on the positive selection of epithelial marker positive cells and then investigated the expression of markers including ASPGR using cytospinning and immunofluorescence to investigate the expression of epithelial and mesenchymal markers and changes of these markers during treatment (Li et al., 2014, Nel et al., 2013). Alternative studies have also used in situ molecular hybridisation to detect genetic aberrations in CTCs including TP53 deletion and HER-2 amplification (Xu et al., 2011).

3.1.2 HCC Biomarkers

The use of clinically-implemented biomarkers in HCC is limited. Serum AFP is routinely measured as part of surveillance programmes for at-risk patients with cirrhosis and also throughout a HCC patient's treatment and follow-up. However, it lacks sensitivity (50-65%) and specificity (76-91%); sometimes not being elevated until late stage disease and in some cases not at all (Sherman, 2001). CA19-9 is another serum marker that is associated with worse prognosis in HCC, but it is also elevated in other cancer types including pancreatic, oesophageal and colorectal cancer (Hsu et al., 2015).

Previous work within our group has indicated that tumour tissue expression the enzyme central to DNA damage repair by NHEJ, namely DNA-PK, in patients with HCC may be a predictor of response to HCC treatment with TACE (Cornell et al., 2015). DNA-PK expression was assessed in liver biopsies from patients receiving TACE using IHC. Patients with a DNA-PK expression higher than median had shorter TTP compared to those that exhibited lower levels of DNA-PK expression (18.8 months vs 31.4 months; p=0.54) (Cornell et al., 2015). Consequently we hypothesised that if DNA-PK was detectable on CTCs in peripheral blood it may be a less invasive predictor of response to TACE.

Other selected biomarkers of interest included the heparin sulphate proteoglycan GPC3 and its modifying endosulfatase, SULF2. From a diagnostic perspective GPC3 is highly specific for HCC. Although it lacks sensitivity, serum levels do not correlate with AFP and it was hoped that used in combination, serum AFP and GPC3 might detect a higher proportion of patients with HCC (Capurro and Filmus, 2005). Also, GPC3 exploration as a treatment candidate targeting GPC3 tumoural overexpression has led to the development and clinical trials of monoclonal antibody GC33 (Zhu et al., 2013). If it was possible to detect tumour specific GPC3 in the blood, it may be possible to identify patients that might benefit from such a treatment should it progress to clinical use. SULF2 removes sulfate groups from GPC3, facilitating its role as a morphogen promoting Wnt signalling. SULF2 has an oncogenic role in HCC and is found to be up-regulated in 57% (79/139) of cases of HCC at the RNA level (Lai et al., 2008). Furthermore, research has shown that targeting SULF2 may repress its oncogenic effects in HCC (Lai et al., 2008). Exploration of this alternative biomarker may shed light on HSPG modification of cancer cell growth, but may also be used as a biomarker for stratifying with anti GPC3 or anti SULF2 treatments.

Following the optimisation of a suitable method of CTC detection in blood **Chapter 2**, it was necessary to see if this method was applicable to detect CTCs in HCC patient samples. Initially samples were collected from a range of patients with varying treatment regimens and clinical parameters. Samples were collected throughout method optimisation in order for any alterations in sample processing to be tested on patient

samples. Two groups of control patient samples were collected: healthy volunteers and cirrhotic patients to ensure that events observed were not a normal occurrence or as part of an immune response. A definition was proposed for the identification of CTCs in patients samples based on the CellSearch definition of EpCAM+/CK+/DAPI+/CD45-. Objects that did not fit these criteria but appeared to have a cellular morphology and were larger in size compared to a WBC and CD45-ve were also recorded and will be discussed.

Two further biomarkers – c-Met and pERK **Section 1.3.7-1.3.8**– were also explored in a small cohort of 14 HCC patients as part of a Masters project carried out by M. M^{c}Cain. C-Met was selected on the basis that HCCs with altered c-Met expression resulted in a more aggressive phenotype and poorer prognosis (Kaposi-Novak et al., 2006). Due to altered C-Met expression in multiple cancers, c-Met is an attractive therapeutic target and early trials of tivantinib (ARQ197) in patients with high c-Met expression had longer TTP than those receiving placebo (Santoro et al., 2013a, Santoro et al., 2013b).

Aberrant Ras/ERK activity has been identified in ~5% of HCC for which pERK can be used as a marker since it leads to the activation of transcription factors of genes responsible for proliferation and survival (Llovet and Bruix, 2008, Newell et al., 2009, Zucman-Rossi, 2010). Previous research has also shown that activation of the RAS signalling pathway in a murine model of hepatocarcinogenesis led to increased tumour number and features of SH-HCC; in human HCC pERK expression in 20 HCC tissue biopsies, 85% had elevated pERK expression (Whitehead et al., 2015). From a therapeutic perspective refametinib (BAY86-9766), a MEK1/2 inhibitor is being trialled in HCC patients with unresectable or metastatic disease with a known KRAS or NRAS mutation; demonstrating the potential for blood tests to be able to stratify patients for therapy (Schmieder et al., 2013).

3.1.3 ctDNA in HCC

Detection of ctDNA in HCC has largely focussed on quantification and comparing the expression of biomarkers in HCC patients compared to controls **Section 1.2.9**. The detection of ctDNA may be improved in terms of specificity using mutation-specific PCR (Anker et al., 1999). In this chapter, in addition to pERK expression being explored in HCC patients, a method of ctDNA extraction from stored HCC plasma samples was developed in order to investigate the KRAS mutational status in patients with HCC that had developed tumours on a background of NAFLD/NASH.

3.1.4 HCC cell lines

A panel of 6 HCC cell lines (HepG2, Hep3B, Huh-7, PLC/PRF/5, SNU182 and SNU475) were assessed for the expression of classical epithelial and exploratory biomarkers using the Imagestream. HCC cell lines were grown and maintained as described in **Section 2.3.1**. Cells were trypsinised and centrifuged at 1000 x *g* for 5 min. Cell density was determined using a haemocytometer. The cell pellet was washed in PBS then cells were fixed in 1% formalin for 20 min at RT or 5 ml ice cold methanol for 20 min. Cells were spun at 1000 x *g* for 5 min and the fixative was replaced with 1 ml of permeabilisation/wash buffer (BD Bioscience) containing saponin for 1 h at RT. Cells were centrifuged at 1000 x *g* for 5 min and re-suspended in 100 µl of permeabilisation/wash buffer to dilute the appropriate antibodies. Antibodies were added in order, depending on the localisation of the protein of interest. Following antibody staining, cells were re-suspended in PBS and ran through the Imagestream with appropriate laser settings. Single colour controls were also ran alongside to enable compensation between adjacent channels.

3.1.5 Imagestream IDEAS® analysis for assessing the expression of HCC biomarkers in HCC cell lines

HCC cell lines were used to assess the expression of biomarkers of interest. The SJSA1 human osteosarcoma cell line was used as a negative control for the expression of epithelial biomarkers. Cells were discriminated from debris by creating a scatterplot of area vs aspect ratio. Single cells that were used for analysis were gated. A histogram of the gradient root mean square of each image was plotted to identify the population of cells that were in focus. The gradient root mean square feature detects changes in pixel values within an object. Round, single and in-focus cells were then assessed for the expression of biomarkers of interest. The percentage of cells within the final population that were positive for the biomarker of interest were measured. Secondly, the expression

of biomarkers was measured using the mean pixel intensity feature. Data was then exported to GraphPad Prism Software, Version 6 (GraphPad Prism Software Inc, California, USA).

3.1.6 CTC HCC patient cohort

69 patient blood samples were collected alongside 15 healthy volunteer samples and 16 cirrhotic control samples. 16 of the aforementioned control samples were collected and analysed by Dr D. Jamieson and Dr C. Hutton. Patient blood samples were collected from the Newcastle Freeman Hospital with ethical approval obtained through the Newcastle Hepatopancreatobiliary and Gastroenterology Research Tissue Bank. Samples were collected from patients with various disease stages and receiving different treatment regimens. A minimum patient dataset was recorded, including patient age; sex; body mass index (BMI); underlying cirrhosis or not; associated chronic liver disease; mode of presentation; stage at presentation (including Childs-Pugh stage of underlying liver function; and TNM stage of the cancer, as well as the BCLC stage). Liver function features recorded included serum bilirubin, albumin, prothrombin time, as well as the presence of ascites or encephalopathy. Tumour features recorded included tumour number and size, as well the absence or presence of extrahepatic disease and portal vein involvement. Additional data collected included the presence of features of metabolic syndrome and ECOG Performance Status. A record was also made of treatments received or anticipated, and ethical approval permitted the follow up of patients to determine response to treatment, time to progression and survival where possible. Time to progression data was collected by Dr H. Reeves and Dr J. Orr.

3.1.7 Patient blood sample collection

Patient blood samples were collected in either EDTA tubes (BD Vacutainer) and transported on ice; or CellSave preservative tubes (Janssen Diagnostics) and incubated at RT for cells to fix for a minimum of 1h. Samples were processed as outlined in **Section 2.3.4-2.3.5**. Following enrichment, the pellet of cells were re-suspended into 100 µl of

perm-wash buffer and incubated with appropriate antibodies **Table 2.2-Table 2.3**. Following antibody incubation, cells were spun at 470 x *g* for 5 min then re-suspended in 50 µl of PBS prior to being ran though the Imagestream with the suitable template that enabled collection of everything that was bigger than an Amnis SpeedBead.

3.1.8 Imagestream IDEAS Analysis for CTC detection

Single colour conjugate controls were collected to create a compensation matrix to account for spectral overlap between different fluorescent antibodies. Analysis was carried out on the basis of cell area. A brightfield analysis mask was created based on the standard brightfield mask and eroded by 3 pixels to give a tighter fit to the brightfield image. A feature was then created from this mask to enable area calculations and further analysis. A histogram of brightfield area with the modified brightfield mask was created. Single WBCs formed a single peak and then anything that was larger in area compared to a WBC was gated into a separate population. This population was then assessed for the presence of CTCs. CTCs were identified on cell morphology, the positive expression of biomarkers included in the panel and the absence of positive CD45 staining. From this a further population of CTCs and objects that appeared to have a cellular morphology and nuclear stain but did not positively express the biomarkers tested was created.

3.1.9 Statistical Methods

Statistical analyses were performed using SPSS, version 21 (SPSS Inc, Chicago, USA) and GraphPad Prism Software, version 6 (GraphPad Prism Software Inc, California, USA). Pearson or Spearman correlations were used to assess bivariate correlations for parametric and nonparametric data respectively. The t-test or Mann-Whitney test was used to investigate differences between groups where data was parametric or non-parametric respectively. Pearson Chi square was used to assess differences between categorical variables and where data sets where small the Monte-Carlo approach was adopted. Survival differences were assessed using the Kaplan-Meier and Log-Rank method. To identify factors associated with survival, initially univariate Cox proportional

hazards-regression model was used. Factors that were identified as significant in the univariate analysis ($p<0.05$) were then entered into a multivariate analysis to determine factors that were independent predictors of prognosis.

3.1.10 DNA extraction from cell lines

DNA was extracted from a panel of six HCC cell lines using the Qiagen mini DNA kit as per manufacturer's protocol. Briefly, 5 x 10^6 cells were trypsinised and loaded onto a DNA binding column. DNA was then subject to a series of wash steps to desalt and purify extracted DNA prior to elution.

3.1.11 DNA extraction from FFPE sections

DNA was extracted from slides of formalin fixed paraffin embedded (FFPE) sections from colorectal cancer patients. Tumourous or normal areas of FFPE liver resections slides were scraped using a needle and added to a centrifuge tubes. Tissue sections were washed twice in xylene to remove wax from the embedding procedure. Samples were then subject to two washes in ethanol. First, tissue was incubated with 100% ethanol for 15 min at RT followed by centrifuging for 2 min at full speed. This step was repeated with 75% ethanol. A proteinase k digestion (1mg/ml in TE buffer, 0.5% Tween-20) step was carried out for 24 h at 55 ^oC. 500 µl of phenol:chloroform:isopropanol (25:24:1) was added to the tissue and the sample was mixed by vortexing. The suspension was then centrifuged at 12,000 x *g* for 5 min and the upper layer of fluid was transferred to a sterile centrifuge tube. One volume of chloroform was added to the sample and mixed by vortexing. The sample was then centrifuged once more at 12,000 x *g* for 5 min and the upper layer transferred to a clean centrifuge tube. 0.3 M sodium acetate was added at a 0.1 of the volume of sample and mixed by vortexing. 1 volume of isopropanol was added to the sample and incubated at -20 ^oC overnight. The sample was then centrifuge at 12,000 x *g* at 4 ^oC to pellet the precipitated DNA and then washed with 75% ethanol. DNA was left to dry in a hood and then dissolved in 50 µl of distilled water.

3.1.12 ctDNA extraction from HCC plasma samples

HCC patient plasma samples were collected with ethical approval obtained through the Newcastle Hepatopancreatobiliary Tissue Biobank. DNA was extracted from HCC plasma samples using the DNA Blood Mini kit (Qiagen®) as per protocol. Briefly, plasma samples were thawed and 200 µl aliquots were subjected to a proteinase K digest for 10 min at 56 ^{o}C. Samples were then vortexed with an equal volume of ethanol to enable DNA to bind to the spin column. The sample was then loaded onto a QIAmp Mini spin column and spun at full speed for 1 min. The eluate was collected and discarded. Subsequent washes were carried out with buffers AW1 for 1 min at 6000 x g and AW2 for 3min at full speed. The DNA was then eluted by loading 50 µl DNAse free water onto the column, incubating for 5 min and then centrifuging at 6000 x g for 1 min. The eluate was added back on to the column and spun again at 6000 x g for 1 min.

3.1.13 Determination of DNA concentration

DNA extracted using the above described methods 1.3.1-1.3.3 was stored at -80oC. Concentration (ng/µl) was determined using the NanoDrop® ND-1000 spectrophotometer (Nanodrop technologies, USA). A260/A280 ratios were recorded to assess purity.

3.1.14 KRAS Sequenom MassARRAY iPLEX

DNA samples extracted from HCC plasma samples were sent to NewGene Ltd, Newcastle-upon-Tyne, UK. The KRAS assay was performed using the Sequenom MassARRAY iPLEX platform. This method is a high throughput single nucleotide polymorphism (SNP) platform that utilises matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry to resolve.

Gene	Mutation	Gene	Mutation
KRAS	G12R	KRAS	Q61X
KRAS	G12S	KRAS	Q61P
KRAS	G12C	KRAS	Q61R
KRAS	G12A	KRAS	Q61L
KRAS	G12D	KRAS	Q61H
KRAS	G12V	KRAS	Q61Q
KRAS	G13R	KRAS	Q61H
KRAS	G13S	KRAS	A146P
KRAS	G13C	KRAS	A146T
KRAS	G13A	KRAS	A146S
KRAS	G13D	KRAS	A146G
KRAS	G13V	KRAS	A146E
KRAS	Q61E	KRAS	A146V
KRAS	Q61K	BRAF	V600E

Table 3.1 List of mutations explored using the Sequenom MassARRAY iPLEX platform.

3.2 Aims

- To explore the presence and characteristics of CTCs in HCC patient blood samples using the Imagestream using an optimised biomarker panel.
- To explore correlations between the presence of CTCs with clinical parameters; including tumour characteristics, time to progression and survival.
- To develop an assay to detect ctDNA in HCC patient blood samples and determine KRAS mutational status.

3.3 Results

3.3.1 Newcastle CTC cohort

Blood samples were collected from 69 HCC patients and processed and assessed for CTCs as outlined in **Chapter 2**. The patient set from which blood samples were collected included a range of patients at different stages of disease progression **Table 3.2**. The median patient age for the cohort was 73 years and approximately two thirds of patients (66.7%) had HCC developed on an aetiology of ALD (31.9%) or NAFLD (34.8%). The majority of patients (71%) had liver cirrhosis, with a mean of 1.9 SEM±0.2 nodules and a mean tumour size of 58 SEM±6mm. Few patients had known extra-hepatic disease (13%) and 18.8% had PVT. Most patients had underlying liver disease that was categorised as Child-Pugh A but HCC that was defined as BCLC stage C, attributed to portal vein invasion (18.8% of all patients), the presence of EHD (13% of all patients) or a performance status of >0 (69.6% of all patients). Most samples - 40/69 (58%) - were taken prior to the commencement of treatment but in the post-treatment cases, the majority received loco-regional arterial therapy (with DEB-TACE or SIRT). Supportive care was the second most common HCC management decision.

Cases	All	At diagnosis	Post-treatment
Number of cases	69	40	29
Age (median)	73	74	69
Liver disease			
None	8	6	2
ALD	22	13	9
NAFLD	24	12	12
PBC/AIH	9	3	6
Haemochromatosis	4	4	0
Cryptogenic	2	2	0
Cirrhosis (present)	49	25	24
Tumour number	1.9±0.2	1.9±0.2	1.8±03
Tumour size (mm)	58±6	72±9	38±5
PVT (present)	14	9	5
EHD (present)	9	4	5
Childs Pugh A/B/C	58/8/3	31/6/3	27/2/0
BCLC A/B/C/D	11/5/50/3	4/1/32/3	7/4/18/0
PST 0/1/2/3	21/17/27/4	8/9/1/9/4	13/8/8/0
AFP (median kU/L)	14 (≤1-≥50000)	14 (≤1-≥50000)	18.5 (≤1-≥50000)
Albumin (g/l)	38.9±5.4	38.5±5.3	39.5±5.6
Bilirubin (µmol/l)	18.2±15.4	19.0±15.3	17.2±15.8
Prothrombin time (s)	12.6±2.5	12.9±2.7	12.3±2.3
Encephalopathy (present)	4	2	2
Ascites (present)	6	5	1
Primary Treatment			
Liver transplant	4	2	2
Resection	3	2	1
Ablation	7	3	4
Arterial treatment	27	7	20
Sorafenib	9	7	2
Supportive care	19	19	0

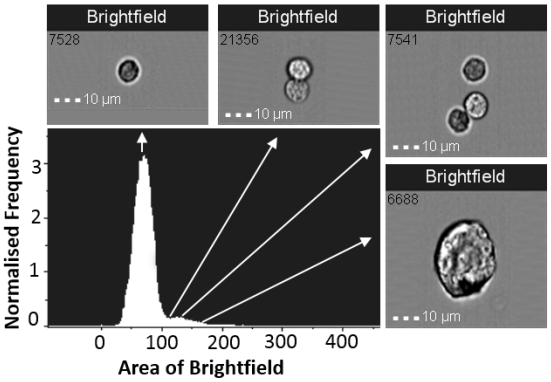
Table 3.2 Demographics of patients in the CTC HCC cohort (n=69). Blood samples were collected from patients during diagnosis, staging and pre- and post-treatment. Ablation treatment was using microwave and aterial treatment was using drug eluting bead-transarterial chemoembolisation (DEB-TACE) or selective internal radiotherapy(SIRT). ALD=alcoholic liver disease; NAFLD=non-alcholic fatty liver disease; PBC=primary biliary cirrhosis; AIH=autoimmune hepatitis; PVT=portal vein thrombosis; EHD=extra-hepatic disease; BCLC=Barcelona Clinic for Liver Cancer; PST=performance status; AFP=alphafetoprotein. ±SEM.

3.3.2 Between 1-1642 CTCs were detected in 65% of HCC patient samples using the Imagestream and CTCs could be detected on the basis of size

Based on data generated during development and optimisation of the CTC assay (**Chapter 2**), since the expression of HCC biomarkers was heterogeneous amongst HCC cell lines, CTCs were initially identified on the basis of one of a panel of positive biomarker expression (CK, EpCAM, GPC3, SULF2, DNA-PK) in combination with a positive nuclear stain and CD45 negativity. Subsequent analyses confirmed that the size of HCC CTCs was considerably larger than WBCs and consistent in size with HCC cell lines **Figure 3.1B**. The mean surface area of biomarker positive CTCs was 362.2 μm^2 SEM±55.5; which was similar to the area of biomarker negative CTCs (345.2 μm^2 SEM±38.4) and HCC cell lines which ranged from the smallest being HepG2 (219.5 μm^2 SEM±2.1) to SNU475 (402.7 μm^2 SEM±2.9). The area of CTCs detected and HCC cell lines was in contrast to the area of WBCs of which the mean was 88.3 μm^2 SEM±0.2. Therefore, by selecting a sub-population of cells on the basis of brightfield area a smaller final population of cells to analyse by eye was selected **Figure 3.1A**. Observers were not blinded to patient status and were aware of whether the sample was a control or from a patient with HCC.

In the Newcastle HCC CTC patient cohort **Table 3.2**, CTCs were detected in 45/69 (65%) of HCC patients. In the control samples, which consisted of 15 healthy volunteers and 16 cirrhotic patients, 0 CTCs were detected. The number of CTCs in HCC patient samples was highly variable with CTC counts ranging from 1-1642 CTCs per 4ml of blood **Figure 3.1C**. In 38 out of the 45 patients that had CTC-positive samples, ≤10 CTCs were detected per 4 ml of blood. A further 5/45 patients had a CTC count between 10 and 50 CTCs per 4 ml of blood. Two patients that had CTC-positive samples had high CTC counts of 197 and 1642 CTCs per 4 ml.

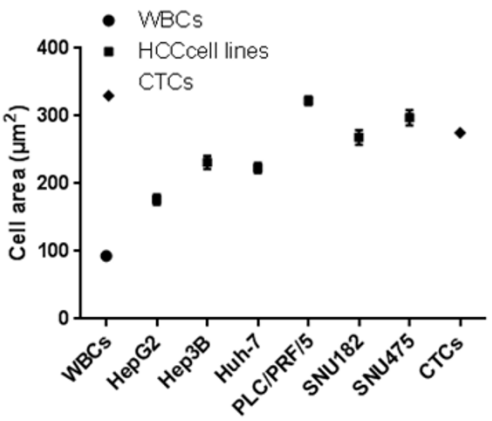
A



B

Cell type	Surface Area (μ^2)	num
WBC	88.3 ± 0.2	8000
HepG2	219.5 ± 2.1	1130
Hep3B	243.0 ± 1.5	2182
Huh-7	268.7 ± 2.1	1777
PLC/PRF/5	244.4 ± 1.3	1678
SNU182	388.9 ± 1.8	2343
SNU475	402.7 ±2.9	1850
CTC	284.2 ± 5.8	380
Macrophages	895.9 ± 41.9	100

C



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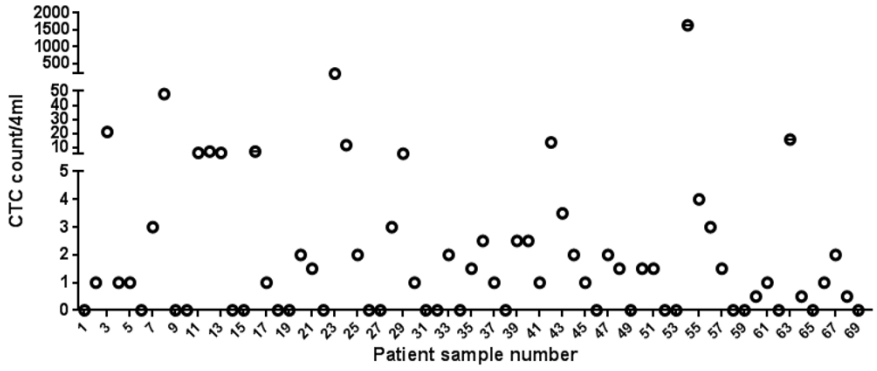


Figure 3.1 A. CTCs could be discriminated from WBCs on the property of brightfield area. C. Area of CTCs was greater than that of WBCs and comparable to HCC cell lines. D. CTC count in HCC patient samples was highly variable with a range of 1-1642 CTCs per 4ml of blood.

3.3.3 Heterogeneity in biomarker expression in HCC CTCs

The biomarker panel for the characterisation of HCC CTCs was initially performed using cell lines. Antibodies and fluorophores were chosen based on the localisation of the protein and to minimise spectral overlap. The final antibody panel included epithelial biomarkers CK and EpCAM to enable comparisons to be made with the CellSearch system reported data. AFP was included since it is the only clinically validated biomarker in HCC. Furthermore, one of the aims of the project was to include biomarkers that had potential clinical relevance. GPC3, SULF2 and DNA-PK were also included since evidence suggest that these biomarkers may be useful in terms of diagnosis and treatment stratification.

Preliminary experiments in HCC cell lines demonstrated the heterogeneity in biomarker expression suggesting that an optimal biomarker panel would include a combination of biomarkers in order to detect and characterise CTCs in HCC patient samples. Although it was later observed that CTCs were larger in size in comparison to WBCs it was noted that there was heterogeneity amongst biomarker expression in HCC CTCs. In cases where CTCs were detected, 37% demonstrated positive expression of one or more biomarkers included in the panel; whereas in a further 28% of cases, the presence of cells that were nucleated, larger in size to WBCs, CD45 negative and did not stain positively for any of the biomarkers included in the panel were noted. This may indicate that these cells may be a sub-type of CTCs that had a different phenotype compared to other CTCs that did positively express the biomarkers of interest; for example cells that had undergone EMT, although this is only speculation. In CTCs that did positively express biomarkers of interest, CK was the most frequently expressed biomarker (29%), followed by DNA-PK (24%), AFP (20%), EpCAM (18%) and GPC3 (12.5%). SULF2 was not detected in any CTCs found in HCC patient samples.

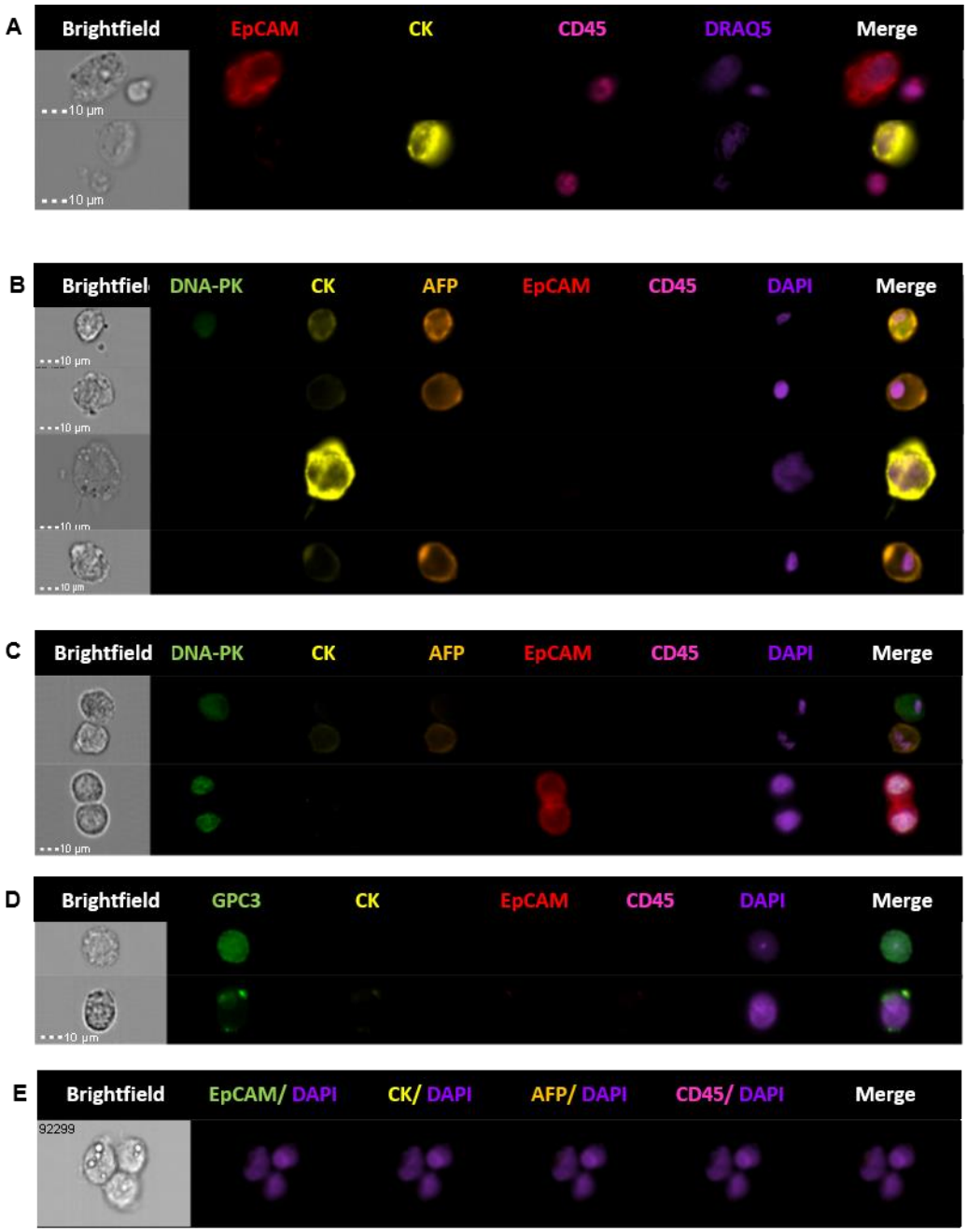


Figure 3.2 Heterogeneity amongst biomarker expression in HCC CTCs. A. CTCs expressing either EpCAM or CK with CD45-posiitve WBCs in the same frame indicating size difference. B. CTCs expressing CK in addition to AFP and DNA-PK. C. Doublets of CTCs expressing DNA-PK or AFP and CK or DNA-PK and EpCAM. D. GPC3-positive CTCs displaying cytoplasmic and membranous expression. E. A triplet of biomarker-negative CTCs, distinguished on cellular morphology, size and DNA content.

3.3.4 CTCs observed travel in the peripheral blood individually and as aggregates with CTCs and leucocytes

In addition to CTCs travelling singularly in the blood, it was also observed that in 14/45 (31%) of CTC-positive patients CTCs travelled together as doublets or aggregates **Figure 3.3A-B**. In 11/14 of cases where CTCs were observed travelling together, CTCs were observed as doublets, while in 5/14 cases, CTCs were observed as aggregates (>2 CTCs per cluster).

Another rare observation made was that of white blood cell interaction with CTCs **Figure 3.3C**. The clinical significance of this observation is not known but in one case where this observation was made in a pre-transplant patient, follow-up data showed that the patient did not have disease recurrence during the follow up period of 33 months post-transplant.

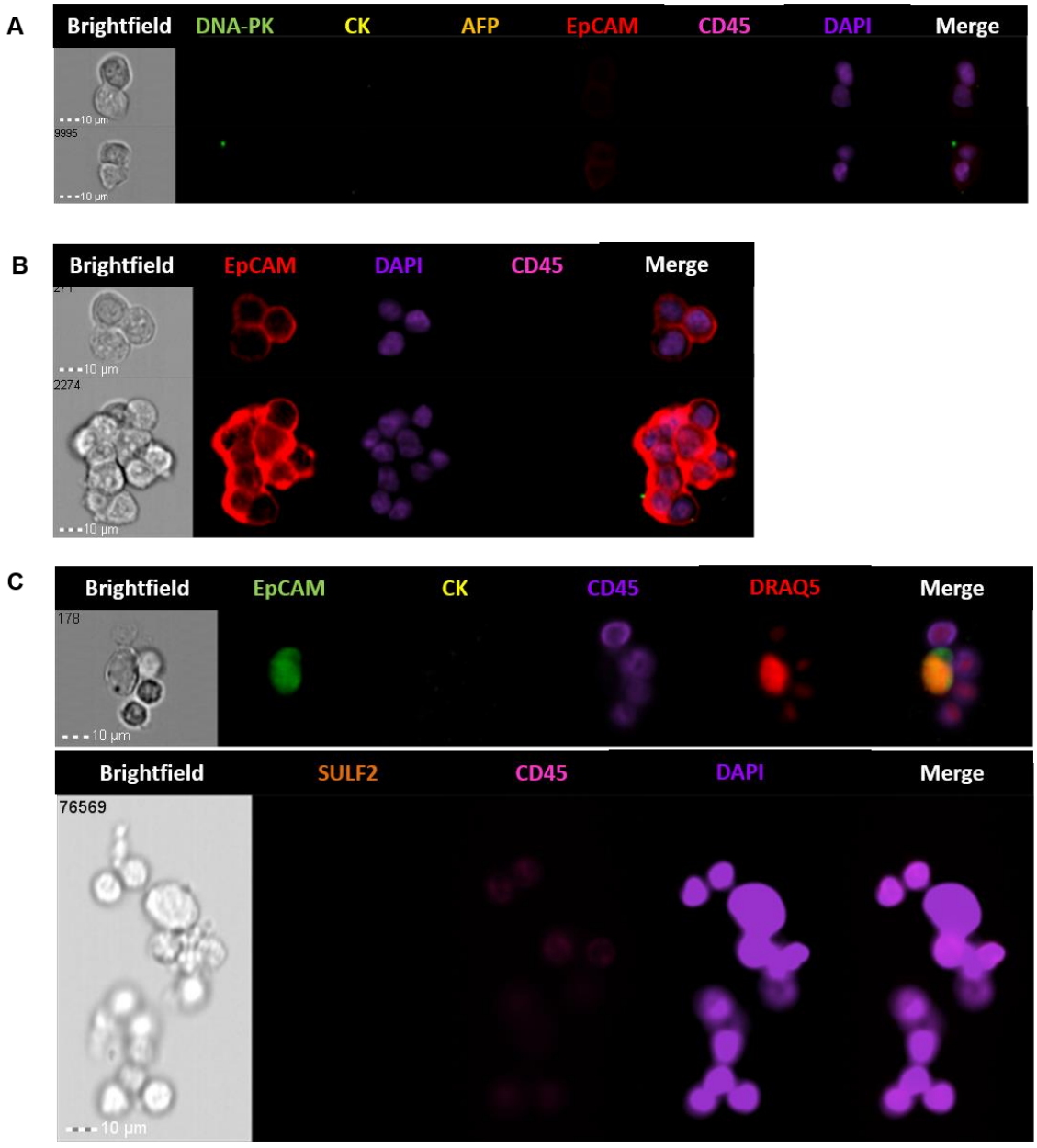


Figure 3.3 A. Doublets of CTCs were observed in 11/45 CTC-positive samples. B. Aggregates of CTCs (>2 CTCs per cluster) were observed in 5/45 CTC positive samples. C. Leucocyte interaction with CTCs was also observed.

3.3.5 Other observations in HCC patient samples included dividing cells and cells with a macrophage phenotype

One of the points up for discussion in CTC research is whether or not the CTCs are viable are capable of forming metastatic lesions. Instances of cells that appeared to be dividing were apparent **Figure 3.4A**. Once again, the clinical significance of this phenomena is not known due to the low number of instances.

A frequent observation in both control samples and HCC patient samples was that of cells with a macrophage-like morphology **Figure 3.1B**. These cells often stained positively for the biomarkers of interest, sometimes being CD45 negative and sometimes appearing CD45 positive. It is unclear whether or not these are definitely macrophages or damaged cells but they were certainly larger in size compared to WBCs and CTCs.

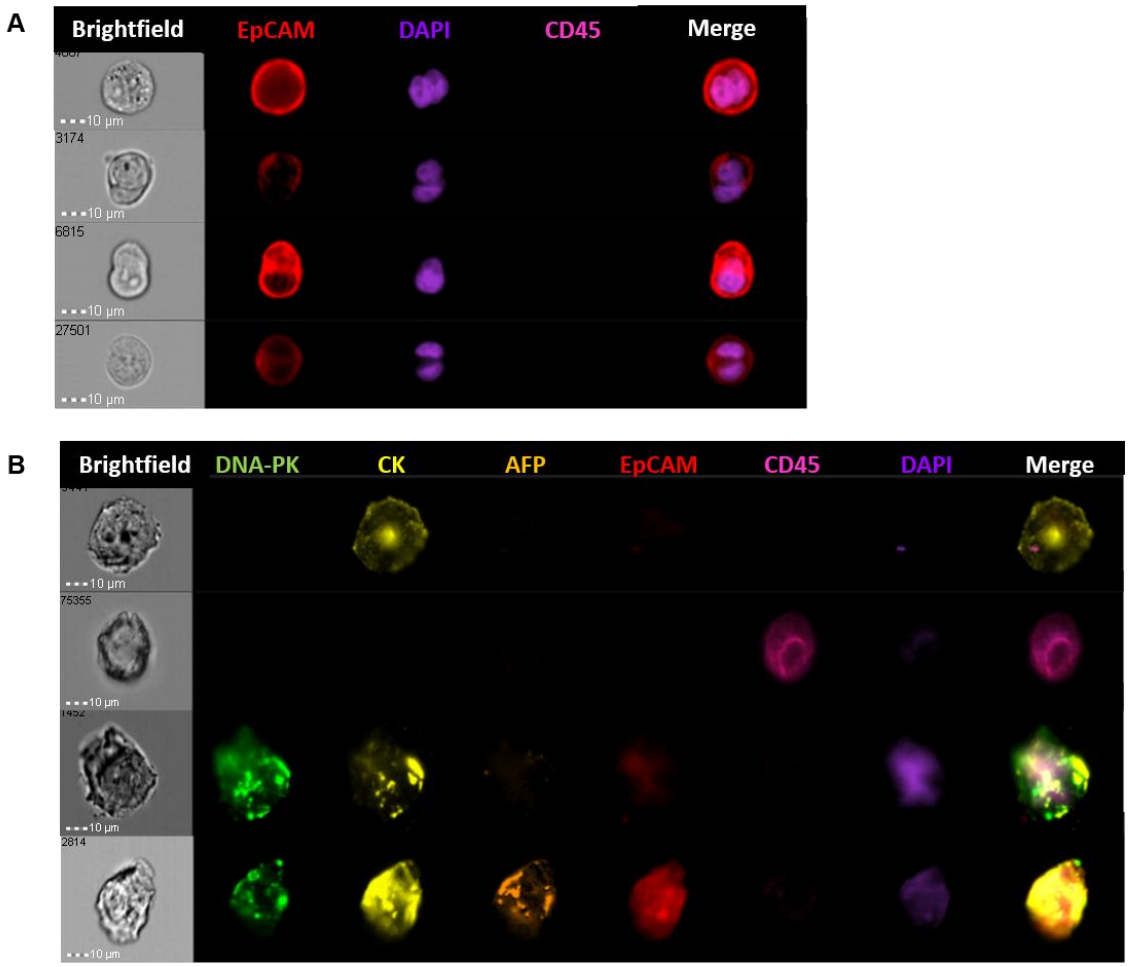


Figure 3.4 A. EpCAM-positive CTCs that appeared to be dividing. B. Cells that were nucleated, large in size and picked up multiple dyes had a morphology consistent with macrophages.

3.3.6 Imagestream CTC count was associated with advanced HCC stage

Samples were collected from 69 HCC patients at varying stages of disease and treatments. Exploration of correlations between CTC number and clinical parameters were explored using bivariate correlations. CTC number positively correlated weakly with features identified with a worse prognosis; including size of the largest tumour (0.291, p=0.015) and presence of hepatic encephalopathy (0.336, p=0.005) Spearman's Rho test. 41/65 HCC patients without hepatic encephalopathy had a median number of 1 CTC per 4ml; compared to the 4/4 patients with encephalopathy where the median number of CTCs detected was 10.

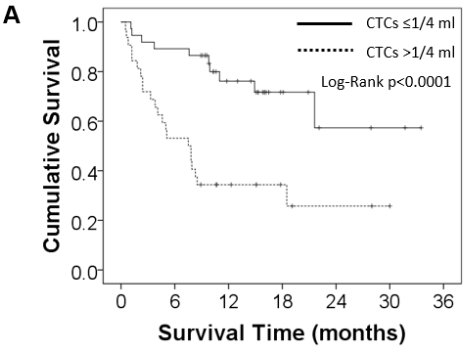
Regarding the biomarker positivity of CTCs detected, correlations were also explored. EpCAM expressing CTCs positively correlated with CK expressing CTCs (0.497, p<0.0001), AFP expressing cells (0.463, p<0.0001) and GPC3 positive cells (0.405, p=0.024). The strongest correlation was observed between CK and AFP positivity on HCC CTCs (0.786, p<0.0001). Correlations between individual biomarkers and HCC disease stage were not significant – possibly due to the small number of cases.

Collectively, numbers of biomarker-positive CTCs had a weak, positive correlation with encephalopathy (0.331, p=0.005) and there was a trend towards association with tumour size (0.236, p=0.05). There was also a weak inverse correlation between the presence of biomarker positive CTCs and serum albumin (-0.354, p=0.003). Biomarker negative cells were weakly associated with the presence of PVT (0.348, p=0.004) Spearman's Rho. Also, there was an indication of an association between biomarker negative CTCs and neutrophils (0.240, p=0.049) in blood counts taken on the same day that the CTC blood sample was collected.

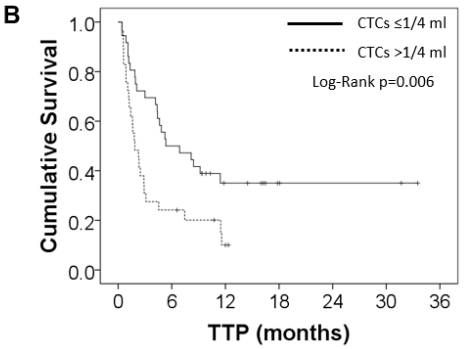
3.3.7 The presence of CTCs in HCC patients is indicative of a shorter survival time

Follow-up data from the Newcastle HCC CTC cohort was collected where possible, including time to radiological or clinical progression and survival. Initially, variable affecting survival were identified in a univariate Cox regression analysis **Table 3.3A**. Factors that were identified as being significantly associated with survival included: number of CTCs per 4ml ($p=0.043$); tumour size ($p<0.0001$); presence of PVT ($p=0.0002$) and presence of EHD ($p<0.0001$).

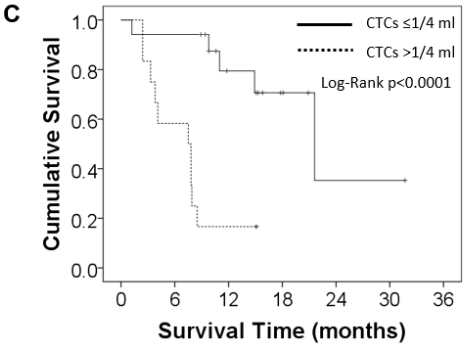
In multivariate analyses **Table 3.3B-C,** CTCs, presence of PVT/EHD were independent of each other. The presence or absence of CTCs was not significantly associated with survival ($p=0.127$). However, when patients were divided into groups based in whether they had ≤1 CTC per 4 ml (n=37), compared to those that had >1 CTC per 4 ml (n=32) notable differences in survival were observed. Receiver operating characteristic (ROC) curve analysis was performed to determine a threshold of >1 (optimal cut-off 1.250, sensitivity 68.75%, specificity 72.97%, likelihood ratio 2.544). Kaplan-Meier regression analysis and the Log-rank test were performed **Figure 3.5.** The presence of >1 CTC/4ml was a significant, independent predictor of survival in the cohort of 69 HCC patients with patients with >1 CTC/4 ml having a median overall survival of 7.5 months compared to those with a CTC count of ≤1 CTC/4 ml who had a median overall survival of 34 months ($p<0.0001$) **Figure 3.5A**. The threshold of ≤1 CTC per 4 ml remained significant in a multivariate analysis ($p=0.049$, HR 2.34, 95% CI 1.005-5.425) **Table 3.3C**. Looking at only the patients that had received prior treatment (n=29), median survival was also different when comparing groups of patients that had ≤1 CTC/ 4 ml and those that had >1 CTC/ 4 ml (21.6 months vs 7.5 months respectively), $p<0.0001$ **Figure 3.5C**. Time to progression was also longer in patients that had ≤1 CTC per 4 ml compared to those that had >1 CTC per 4 ml detected (median 5.3 months vs 1.9 months) Kaplan-Meier with Log-Rank $p=0.006$ **Figure 3.5B**. This was also true in the post-treatment group (8.4 months vs 1.6 months) $p=0.002$ **Figure 3.5D**.



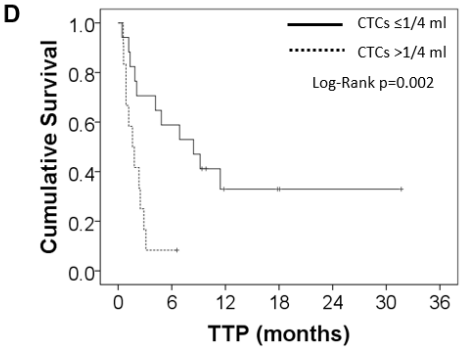
At-risk patients	0	6	12	18	24	30
CTCs ≤1/4 ml	37	33	19	7	3	2
CTCs >1/4 ml	32	17	8	4	2	1



At-risk patients	0	6	12	18	24	30
CTCs ≤1/4 ml	36	18	8	3	2	2
CTCs >1/4 ml	29	7	2	0	0	0



At-risk patients	0	6	12	18	24	30
CTCs ≤1/4 ml	17	16	9	4	4	1
CTCs >1/4 ml	12	7	2	0	0	0



At-risk patients	0	6	12	18	24	30
CTCs ≤1/4 ml	17	10	3	21	1	1
CTCs >1/4 ml	12	1	0	0	0	0

Figure 3.5 Kaplan-Meier survival curves of patients with ≤1 CTC/4ml or >1 CTC/4ml in A. the full cohort of 69 HCC patients and C. Patients that had received any form of treatment (n=29) after which a sample was assessed for the number of CTCs. Time to progressions (TTP) first documented radiological or clinical is shown for the full cohort of patients in B. and for the subset of patients that had received prior treatment in D.

A. Univariate Analysis		B. Multivariate Analysis				C. Multivariate Analysis			
				95% CI				95% CI	
		p	HR	Lower	Upper	p	HR	Lower	Upper
CTC number/ 4ml	0.043	0.025	1.002	1.000	1.003		(Not included)		
CTC >1 / 4ml	<0.0001		(Not included)			0.049	2.335	1.005	5.425
Age (median year)	0.217								
BMI	0.474								
T2DM	0.939								
Cirrhosis	0.643								
No of tumours	0.071								
Size of largest (cm)	<0.0001	0.174	1.005	0.998	1.013	0.065	1.007	1.000	1.014
PVT (cat)	0.002	0.005	3.568	1.467	8.679	0.023	2.575	1.140	5.818
EHD (cat)	0.001	0.003	4.163	1.634	10.605	0.122	1.999	0.831	4.807
Ascites	0.061								
Encephalopathy	0.183								
AFP (median)	0.065								
Albumin (g/l)	0.120								
Bilirubin (µmol/l)	0.582								
Prothrombin time	0.566								
PST (cat)	0.127								
Treatment (cat)	0.072								
BCLC stage	0.187								

Table 3.3 Variables that were significant in the univariate analysis (p<0.05) were entered into the multivariate analysis. Where variables were categorical (cat) the comparator variable was that with the best outcome.

3.3.8 pERK and c-MET expression in CTCs in HCC

A small cohort of HCC patients (n=14) were assessed for the presence of CTCs and the expression of the biomarkers pERK and c-Met (M. McCain, Unpublished data). In this cohort, 3/14 patients provided blood samples that tested positive for CTCs – the lower number perhaps partly attributed to the learning curve associated with sample preparation by our MRes student. Out of the three patients that were CTC-positive, one patient had 3 CTCs that were negative for both pERK and c-Met, one patient had 13 CTCs one of which one was c-Met positive and the final patient provided two samples – one pre-TACE and one 24h post-TACE. In the pre-TACE sample, 46 CTCs were counted; 14 of which were pERK positive and 2 of which were c-Met positive. In the post-TACE sample, twice as many CTCs were counted; however none were pERK positive and only one was c-Met positive. This cohort of patients was too small to infer clinical correlations and was not included in the main cohort analysis of HCC patients.

3.4 Development of an assay for the detection of ctDNA in HCC

Although it was possible to detect CTCs in 65% of HCC patients, often the number of CTCs per 4 ml was low. Methods of ctDNA detection have become more sensitive and specificity has improved through the detection of mutational status. Although the key genetic drivers of hepatocarinogenesis remain to be fully defined, activation of the RAS signalling pathway has been identified in patients with HCC developed on a background of HBV, HCV and ALD (Newell et al., 2009). Previous work within the Newcastle Hepatology research group demonstrated that there was increased activation of the RAS signalling pathway in a NAFLD/NASH murine model of hepatocarcinogenesis; leading to the hypothesis that this may be mirrored in HCC patient samples with a NAFLD/NASH aetiology. Additionally, Refametinib (BAY86-9766), a MEK1/2 inhibitor is currently in clinical trials (trial identifier: NCT01915589) for the treatment of HCC patients with unresectable or metastatic disease with a known KRAS or NRAS mutation.

The KRAS iPLEX method has the ability to detect mutant DNA in a background of wt DNA when it is present at a percentage of 5-10%. This assay is more sensitive than Sanger sequencing which is able to detect mutant DNA if it is present in quantities ~20-30%. Currently, the KRAS iPLEX assay is clinically validated to determine the mutational status of KRAS in patients with colorectal cancer using FFPE sections. Mutations in KRAS can lead to its constitutive activation leading to resistance to the EGFR inhibitor Cetuximab which is a first line therapy in patients with colorectal cancer. Our aim was to determine whether or not it would be possible to translate this assay into a blood-based assay in patients with HCC to determine KRAS mutational status using ctDNA.

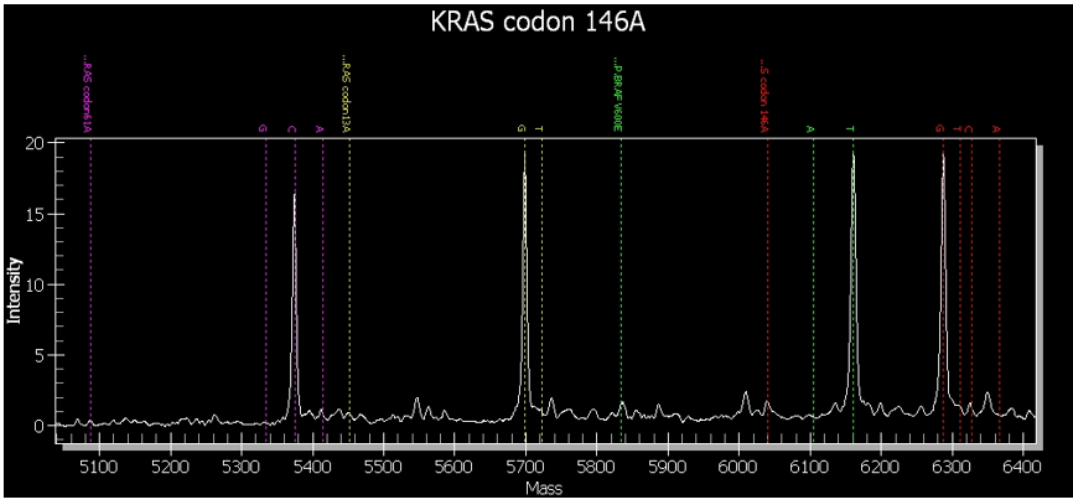
3.4.1 Extraction of ctDNA from frozen plasma samples

In order to determine the suitability of the iPLEX KRAS assay for use with ctDNA extracted from blood, DNA was extracted from three different sample types: FFPE sections, plasma and cell lines. DNA from a colorectal cell line (SW480) - with a known KRAS mutation in codon 12 - was included as a positive control; DNA extracted from FFPE section slides from a colorectal cancer patient and plasma from both a healthy volunteer (negative control) and a HCC patient with a large (4.2cm) tumour were tested. DNA concentrations from the aforementioned samples varied. All samples were tested using the KRAS iPLEX assay whereby the tester was blinded to the sample type. Results confirmed the presence of the G12V mutation in the SW480 cell line and all other samples were negative for KRAS mutations included in the assay.

3.4.2 KRAS mutations ~5% HCC patient samples with HCC developed on a background of NASH/NAFLD

In the initial testing, DNA extracted from FFPE sections was used as a comparator to determine if DNA obtained from plasma samples was of a similar quantity and would perform similarly to DNA extracted from FFPE sections in the KRAS assay. In total, 38 HCC patient plasma samples were tested. KRAS mutations were detected in 2/38 (5.26%) HCC patient plasma samples. Both of these mutations were at the same codon (KRAS codon 12A) involving the same base change (G>T), resulting in an amino acid change from glycine to cysteine. To test the specificity of the assay, one of the samples that had a mutation was re-tested. A mutation in the same codon was detected however this time the base change was detected as G>C as opposed to G>T **Figure 3.6**.

A



B

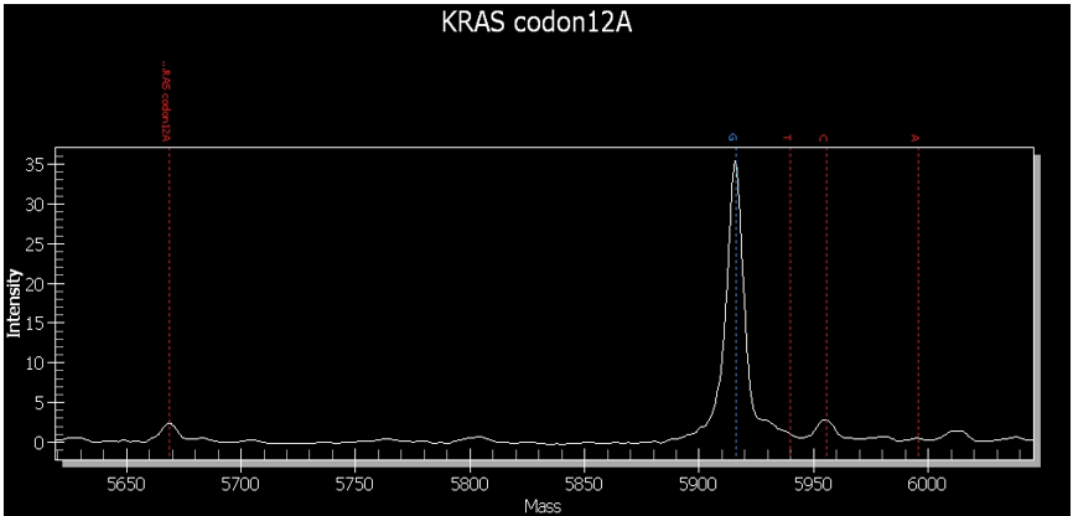


Figure 3.6 DNA extracted from plasma was assessed for the presence of KRAS mutations Table 3.1. A. Wild-type DNA. B. Example showing a mutation of glycine > cysteine in KRAS codon 12.

3.5 Discussion

In the previous chapter, the method of patient sample CTC enrichment and detection was discussed. This was worked up using a panel of 6 HCC cell lines that were heterogeneous for the biomarkers of interest leading in the creation of a panel containing multiple biomarkers. Also, in our patient cohort we were interested to investigate the expression of additional biomarkers including: DNA-PK, GPC3 and SULF2. Overall detection of CTCs in this HCC patient cohort was 65% compared to 0% in the control samples. Taking into account only epithelial-positive CTCs, results were comparable with other studies (Schulze et al., 2013, Morris et al., 2014). Application of a size criterion also identified CTCs that were positive for other biomarkers included in the panel and also identified a population of CD45-negative, nucleated cells that were negative for all biomarkers included in the panel. This suggests that CTC detection platforms that are reliant on epithelial markers may be under-detecting a proportion of CTCs that are epithelial marker negative – possibly as a result of undergoing EMT. Unfortunately, one of the limitations of the method described above is that cells are not viable at the end of the process so it is not possible to perform further downstream analysis on these cells.

Using the Imagestream as a method of CTC detection also led to the observation of some other interesting objects that would have been likely to have gone unnoticed using other forms of CTC detection. In several HCC patients, CTCs appeared to be travelling in clusters. This phenomenon has already been reported by researchers using the ISET technique and it has been suggested that clusters of cells may be more likely to be able to form metastases at distant sites (Vona et al., 2000). It is still not known as to whether this is a true phenomenon or if it is an artefact of the methods used. CTCs with leucocyte interaction were also observed which may be indicative of an immune response towards CTCs, eradicating them from the blood resulting in such CTCs being unable to form metastatic lesions. However, a counter argument to this is that CTCs may use leucocytes as a protective mechanism to prevent immune evasion (Alix-Panabieres and Pantel, 2014). Larger cohort studies would be required in order to infer clinical relevance of these observations.

The clinical utility of CTCs in HCC still remains to be determined but results of this study suggest that CTCs may have a prognostic role in HCC management. Factors that were significant predictors of prognosis included size of largest tumour, EHD and PVT as well as CTC count >1/ 4 ml. In this cohort of HCC patients, the data presented in this chapter demonstrated that the presence of >1 CTC/4ml was an independent predictor of survival and TTP in the whole cohort of patients and in the sub-cohort of patients that had samples assessed following treatment. Although in this cohort of patients, none of the control samples were CTC-positive; previous studies that have looked into the presence of CTCs in HCC using the CellSearch system have classified a level of ≥1 CTC as being a positive result.

Biomarker positive CTCs weakly correlated positively with the size of the largest tumour and hepatic encephalopathy and inversely correlated with serum albumin levels; all of which are associated with worsening HCC stage. In terms of biomarker expression on CTCs, there was strongest correlation between cytokeratin and AFP positive cells but there were also trends between epithelial biomarkers EpCAM and CK as well as EpCAM with AFP and GPC3. Biomarker negative CTCs correlated with neutrophil and platelet counts from blood samples taken on the same day. Studies have indicated that neutrophils have a tumourigenic role in HCC (Piccard et al., 2012, Sionov et al., 2015, Xu et al., 2014). Platelets may also have a tumorigenic role with studies suggesting that they may facilitate EMT and form a “protective cloak” around CTCs enabling them to evade the immune system (Plaks et al., 2013, Labelle et al., 2011).

Isolation of cfDNA from HCC plasma samples was briefly investigated to determine if this may be a suitable method to obtain molecular information from the tumour and potentially have a role in stratifying patients for therapy. The method employed to detect KRAS mutations in ctDNA from HCC samples is currently a clinically validated method for use with FFPE sections from patients with CRCa. The KRAS iPLEX method has the ability to detect mutant DNA in a background of wt DNA when it is present at a percentage of 5-10%. This means that it is more sensitive than Sanger sequencing which is able to detect mutant DNA if it is present in quantities ~20-30%. Although initial testing showed that it was suitable for use on patient plasma samples, in the samples that were tested for the presence of KRAS mutations, in some cases single SNPs failed and in one of the patients where a mutation was detected, on re-

testing the base had changed raising issues about specificity despite this being a clinically validated assay for patients with colorectal cancer.

Ideally, this assay would have been developed using a mutation that was highly prevalent in patients with HCC. For example, mutations in the TERT promoter region have been recognised in ~59% of patients with HCC (Nault et al., 2013). Attempts were made to implement the detection of mutations in this region into a blood-based assay; however due to the high GC content of the promoter region attempts at primer design and assay optimisation failed. Another option could be to use the Ultraseek which is a more sensitive method than the iPLEX method, with the ability to detect ≤1% of mutant DNA in a background of wtDNA. Furthermore, the Ultraseek provides the options of testing for a panel of oncogene mutations. However, this method is much more costly. An alternative method to improve the detection of KRAS mutations in patient plasma samples may be to enrich the DNA prior to running the KRAS iPLEX.

3.6 Conclusions

- 1-1642 CTCs were detected in 65% of HCC patients using the Imagestream. Biomarker expression on patient CTCs was heterogeneous, with some cells negative for all biomarkers included in the panel.
- The presence of >1 CTCs per 4 ml of blood compared to ≤1 CTC per 4 ml of blood indicated a worse median survival time in HCC patients (7.5 months vs 34 months, p>0.0001) and shorter TTP (1.9 months vs 5.3 months, p=0.006). These differences were also observed in the post-treatment group; patients with >1 CTC had shorter median survival compared to those with patients who had ≤1 CTC per 4 ml (7.5 months vs 21.6 months, p<0.0001) and TTP (1.6 months vs 8.4 months, p=0.0002).
- CTC detection using the Imagestream also enables observation of leucocyte-CTC interactions, tumour-associated macrophages and CTC clusters although the clinical significance of these phenomena is unknown.
- It is possible to isolate ctDNA from HCC plasma samples but in order to improve this assay it would be necessary to test for a mutation that is more prevalent in human HCC, use a pre-enrichment step for ctDNA or use a more sensitive method of mutant DNA detection.

Chapter 4. Circulating immune cells and Hepatocellular Carcinoma

4.1 Introduction

Innate immune cells contribute to inflammation, which is recognised as an enabling characteristic for the other hallmarks of cancer (Hanahan and Weinberg, 2011). In hepatocellular carcinoma (HCC), it is estimated that approximately 90% of cases develop within an environment characterised by chronic inflammation (Nikolaou et al., 2013). Furthermore, inflammatory cells are common in the tumour microenvironment, which is comprised of numerous non-malignant cells with tumour-promoting properties, with signalling and communication recognised between the malignant and non-malignant components (Balkwill et al., 2012, Hanahan and Coussens, 2012). Remarkably, it has been estimated that over 50% of the total tumour mass may be comprised of non-tumour cells and stromal tissues (Balkwill et al., 2012). Presently, although inflammatory cells exist in pre-malignant tissues and tumour masses, and the tumorigenic potential of inflammation has been established, the individual roles of inflammatory cells and crosstalk between these cells and the tumour cells in the tumour microenvironment remains to be fully understood. It is suspected, however, that the tumour microenvironment could be an important factor to consider in patients with cancer, possibly associated with progression and prognosis, but also response to treatment (Balkwill et al., 2012).

4.1.1 Neutrophils

Circulating immune cells are commonly called ‘white cells’, as opposed to red blood cells carrying haemoglobin, with the most abundant being the neutrophil. Neutrophils play an important role in the activation and regulation of both innate and adaptive immune responses (Mantovani et al., 2011). Although neutrophils have an important role in the immune response to infection, they can be retained in tissues such as the liver, where their excessive activation, characterised by the release of ROS, has the potential to induce liver damage (Jaeschke, 2006). Associations with viral hepatitis, ALD and NAFLD have been reported, as have associations with progression to fibrosis and cirrhosis. Attempts to reduce the number of

neutrophils has been considered as an immunotherapy, presently limited to patients with HBV and HCV (Xu et al., 2014).

Notably, emerging literature supports paradoxical roles for neutrophils in patients with cancer, possibly related to their phenotype rather than actual numbers in either tissues or the circulation. Akin to tumour associated macrophages (TAMs), tumour associated neutrophils (TANs) have been recently described as potentially having either an anti-tumorigenic (N1) phenotype or a pro-tumorigenic (N2) phenotype. While neutrophils attraction or retention in the liver may be chemokine driven e.g. CXCR2, their phenotype is thought to be further influenced by the tumour microenvironment, where TGF-β is an important effector molecule leading to the switch of neutrophils to a pro-tumorigenic (N2) phenotype (Xu et al., 2014). This switch is not necessarily restricted to the liver. In a study performed by Sagiv and colleagues, distinct populations of circulating neutrophils were described, differentiated on the basis of cell density with cancer patients having a significant increase in the number of low-density neutrophils (Sagiv et al., 2015). N2 neutrophils may have multi-faceted tumorigenic roles, including promotion of tumour growth; evasion of apoptosis; promotion of genetic instability; angiogenesis; invasion, and metastasis, (Piccard et al., 2012, Sionov et al., 2015).

4.1.2 Lymphocytes

The roles of circulating and tissue lymphocytes is an active area of research, but the complexities remain poorly understood. Contrary to neutrophil studies, an increased number of TILs – although rare in HCC - is considered a good prognostic indicator and associated with decreased risk of recurrence in resection patients (Shirabe et al., 2010, Wada et al., 1998). Furthermore, in a randomised trial where 76/150 patients were subjected to immunotherapy in the form of activated lymphocyte infusions post-operatively, their time to recurrence was longer and recurrence frequency decreased by 18% (Takayama et al., 2000). Although there was no significant difference in overall survival of the treated group compared to the control group, recurrences were actively treated, confounding subsequent follow-up. Thus the importance of delayed recurrence should not be lightly dismissed. However, presently we are a long way off assuming all increases in lymphocytes are ‘good'. Increased number of circulating T-regulatory (Treg) cells in the blood of HCC patients compared to controls has

been reported (Ormandy et al., 2005). In another study, the proportion of the Treg population that were TGFβ-positive was increased in HCC patients, rather than total numbers (Unitt et al., 2005). These are small studies and associations with outcome unreported. In a mouse model of inflammation-associated epithelial carcinogenesis, B-cells are required for malignant transformation (de Visser et al., 2005) and there have been reports of an increased number of circulating B-regulatory (Breg) cells in patients with HCC, with higher counts being associated with more advanced disease (Shao et al., 2014). Thus, as in the case of neutrophils, it is likely that lymphocytes can play protective and promoting roles in both tumour initiation and progression.

4.1.3 Platelets

The role of circulating platelets or platelets within tissues in HCC is less well studied and potentially difficult to interpret in patients with liver disease. Circulating platelet counts are often seen to rise in association with inflammation. In patients with cirrhosis and portal hypertension, characterised by hypersplenism and a shorter platelet life expectancy, platelet counts are typically lower, and any relative increase potentially masked. Generally, however, platelets are proposed to have tumour promoting roles. A study carried out by Hynes and colleagues indicated that platelet-activation of both the TGFβ and NFκB-signalling pathways resulted in EMT, increasing invasiveness and metastatic potential of tumour cells (Labelle et al., 2011). Furthermore, it has been suggested that platelets may play a role in cancer metastasis by providing a protective role to circulating tumour cells (CTCs) - shielding them from physical damage and enabling immune system evasion (Plaks et al., 2013).

4.1.4 Circulating inflammatory cells prognostic scores

The ratios of circulating immune cells have been shown to be indicative of prognosis in patients in a range of different cancer types. In HCC, an increased number of neutrophils combined with a decreased number of lymphocytes has often been proposed as the biomarker most indicative of the level of inflammation in patients (Xu et al., 2014). The neutrophil to lymphocyte ratio (NLR) has been shown to have prognostic value in HCC patients undergoing

a range of different treatment regimens. A NLR or ≥5 versus <5 was shown to be a poor prognostic indicator in a cohort of 96 patients undergoing resection (Gomez et al., 2008), as well as in a cohort of 160 patients treated with liver transplantation (Limaye et al., 2013). Chen and colleagues demonstrated that a post-operative increase in NLR was associated with significantly different PFS, as well as OS at 1-, 3-, and 5-years following treatment with radiofrequency ablation (Dan et al., 2013). Thus far, the studies in transplanted patients have been largely limited to those with HCC arising on a background of viral hepatitis (Limaye et al., 2013, Yoshizumi et al., 2013). In the study by Yoshizumi et al, transplanted organs were from living donors, treating cases of recurrent HCC without a size restriction, thus including more advanced cases beyond 'Milan Criteria'. Surgical series also have some limitations, also including patients presenting with more advanced symptomatic disease (Yoshizumi et al., 2013, Gomez et al., 2008). Furthermore, the NLR is an established predictor of cirrhosis irrespective of the presence of cancer (Biyik et al., 2013), and in some studies is not necessarily accounted for in multivariate analyses (Gomez et al., 2008). Thus the true prognostic or predictive value of NLR independent of aetiology and severity of underlying liver disease, at different stages of disease, remains to be validated.

A possible step forward was reported in a recent study taking into account platelets as well as neutrophils and lymphocytes. The research team used these parameters from a standard blood count in a retrospective cohort of 133 resection patients to create the systemic immune-inflammation index (SII) (Hu et al., 2014). X-tile analysis was carried out to determine optimal cut-off values resulting in a group of patients with a low SII ($<330 \times 10^9$) and patients with a high SII ($\geq 330 \times 10^9$) (Hu et al., 2014). A prospective cohort of 123 patients was used to validate the SII. Additional blood samples were also collected from this cohort to assess for the presence of EpCAM and CK-positive CTCs using the CellSearch method. The SII score was compared to both the NLR and platelet to lymphocyte ratio (PLR). In a multivariate Cox regression analysis, the SII was deemed to be a highly significant predictor of OS and relapse-free survival. Correlations with clinical parameters showed that a high SII was significantly associated with large tumours, vascular invasion and the number of CTCs (Hu et al., 2014).

In this chapter, inflammation status in a much larger and heterogeneous cohort of HCC patients has been explored, attempting to validate prognostic scores across all stages of disease, as well as to pinpoint key associations or roles for individual cell types. The patients

included those presenting to our own tertiary referral centre in Newcastle, but also a cohort presenting in Hong Kong. These combined patient cohorts comprised 1168 patients, with the opportunity to assess the impact of different aetiologies, clinical factors and treatment regimens. We have explored the inflammatory status in HCC patients by investigating relationships between circulating inflammatory cells and clinical parameters. Furthermore we have investigated the prognostic value of previously described immune cell ratios in this large patient dataset.

4.2 Aims

- To define the association between the counts and ratios of circulating inflammatory cells and clinical parameters, including tumour stage and markers of liver function, in a test patient cohort.
- To explore the associations of circulating inflammatory cells and scores with survival in the test cohort.
- To investigate if the circulating inflammatory biomarkers retained prognostic value in an independent cohort of patients, with a different predominant aetiology undergoing different treatment regimens
- To explore in a single, combined, larger cohort the value of the candidate inflammatory biomarkers of predicting outcome overall as well as within defined stages or treatment categories

4.3 Materials and Methods

4.3.1 HCC Patient Blood Count Data - Newcastle Cohort

Blood count data was retrospectively collected from a cohort of HCC patients (n=583) presenting at the Newcastle-upon-Tyne Foundation Trust between 2000 and 2010 **Table 4.1**. Laboratory blood data included total white cell count (WCC), neutrophils, lymphocytes and platelets. Serum measurements of AFP, albumin, bilirubin, INR, sodium and creatinine were also recorded. The blood count data used was that collected closest to the date of diagnosis. Clinical parameters collected alongside included patient demographics (age, sex); aetiology; mode of presentation; assessment of underlying liver disease (presence/absence of cirrhosis, Child-Pugh score and grade); tumour characteristics (number of nodules, size, extra-hepatic metastases, PVT, lymph node involvement, ascites, encephalopathy); staging information (TNM, BCLC classification, Okuda, CLIP, ECOG Performance Status) and primary treatment. Current survival status was recorded and overall survival was calculated in months from the date of diagnosis to the date of death (Dyson et al., 2014).

This dataset was used as a training cohort to explore the relationships between circulating immune cells and clinical parameters as well as to determine categorical grouping cut-off values.

4.3.2 Hong Kong Validation Cohort

In order to explore whether or not observed relationships were reproducible in a non-UK based cohort, data from HCC patients with a predominant HBV/HCV aetiology were collected from a consecutive cohort of HCC patients (n=585) presenting to Dr Stephen Chan and colleagues at the Prince of Wales Hospital, Hong Kong, between 1st January 2007 and 31st July 2013.

4.3.3 Combined cohort

Patient data from all three cohorts was re-coded appropriately to form a combined cohort consisting of a total of 1168 HCC patients.

4.3.4 Neutrophil to lymphocyte ratio and SII score

The neutrophil to lymphocyte ratio was calculated by dividing the total number of neutrophils by the total number of lymphocytes.

The SII was calculated as outlined by Hu and colleagues (Hu et al., 2014).

$$\mathrm{SII} = Platelets\left(\frac{Neutrophils}{Lymphocytes}\right)$$

4.3.5 Statistical analysis

All statistical analyses were performed using IBM® SPSS® Satistics for Windows, Version 22.0, IBM® Corporation, Armonk, New York, USA). Comparisons were performed using the Kruskal-Wallis test. Associations with defined parameters were using univariate Cox regression analysis. Variables that were significant ($p<0.01$) in a univariate Cox regression analysis were included in multivariate Cox regression analyses. When including categorical variables in the multivariate analysis, those with the best outcome or lowest stage were used as comparator values denoted by '(first)' unless otherwise stated. Additional survival analyses for plotting daya were performed using the Kaplan-Meier method and Log-Rank test. Bivariate correlation analyses were carried out using Pearson's correlation coefficient for normally distributed parametric data and Spearman's rho for non-parametric data or data that was not normally distributed. Statistical significance was denoted by the following: ns = not significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Subsequently, the Bonferroni correction was also performed to account for multiple comparison. Results are displayed in a table following the initial association testing. For the Bonferroni correction, the p-value ($\alpha=0.05$) was adjusted based

on the number of associations that were tested (n=45). The adjusted p-value = α/45 = 0.001; therefore associations that were significant at the p≤0.001 level were identified.

4.4 Results – Newcastle training cohort

4.4.1 Newcastle cohort description

The Newcastle cohort consisted of mostly males (81%) of which the median age was 69 years old. The commonest aetiologies for CLD were alcohol-related and NAFLD. Nearly three quarters of patients (73%) had underlying cirrhosis and assessment of underlying liver disease scored most patients as Child-Pugh A. On average patients had 2.55 nodules measuring 6.17 mm SEM±0.19, with PVT present in approximately a quarter of patients. EHD was present in a small proportion of patients (16%). Using the BCLC classification, the majority of patients (69%) were classed as BCLC C-D. Most patients received arterial treatment or best supportive care.

4.4.2 Relationship between circulating inflammatory cells, tumour stage and liver function

The classical cancer staging system is the TNM stage, based on tumour number and size, but also invasive characteristics including lymph node invasion and extrahepatic metastases. The details of the HCC TNM staging system are previously described **Section 1.1.9**, as are its shortcomings in patients with underlying CLD and impaired liver function. The BCLC staging system incorporates an estimate of liver function in the form of the Child-Pugh grade, alongside tumour number, size, portal vein invasion and EHD. The numbers of circulating inflammatory cells in each of these systems (TNM, Child-Pugh, and BCLC) were compared.

Patient demographics			
Male/Female (number)	473/110		
Age (median)	69 (range 18 - 93)		
Aetiology			
Unknown/None	114		
Alcohol related	168		
Non-alcoholic fatty liver	127		
HBV/HCV	28/60		
HBV & HCV	-		
PBC/AIH	19/7		
Haemochromatosis	31		
Cryptogenic	24		
Other	5		
Cirrhosis (Absent/Present)	157/426		
Tumour characteristics			
Number of tumours	2.55 ±0.11		
Size of largest tumour (mm)	6.17 ±0.19		
Portal vein thrombosis (Absent/Present)	430/153		
Extrahepatic disease (Absent/Present)	489/94		
Laboratory blood data			
Median AFP (median; kU/L)	27.5 (range 1 – 640,000)		
White cell count (x10^9)	7.41 ±0.21		
Neutrophils (x10^9)	4.82 ±0.12		
Lymphocytes (x10^9)	1.71 ±0.15		
Platelets (x10^9)	198.87 ±5.32		
Neutrophil to lymphocyte ratio	4.11 ±0.19		
SII	855.11 ±46.34		
Albumin (g/L)	35.70 ±0.24		
Bilirubin (µmol/L)	29.16 ±1.61		
INR	1.09 ±0.01		
Sodium (mmol/L)	138.9 ±2.07		
Creatinine (µmol/L)	102.64 ±1.74		
Staging			
ECOG Performance Status		**Child-Pugh**	
0	217	**A**	369
1	160	**B**	132
2	115	**C**	80
3	78		
4	13		
BCLC Stage		**TNM Stage**	
0/A	88	**1**	214
B	94	**2**	106
C	268	**3**	168
D	133	**4**	95
Primary Treatment			
Liver transplant	53		
Resection	31		
Ablation	65		
Arterial treatment	188		
Sorafenib	9		
Supportive care	237		

Table 4.1 Newcastle cohort patient demographics (n=583). Values are shown as number or mean ± standard error unless otherwise stated. HBV=hepatitis B virus; HCV=hepatitis C virus; PBC=primary biliary cirrhosis; AIH=autoimmune hepatitis; AFP=alphafetoprotein; SII=systemic immune-inflammation index; INR=international normalised ratio; ECOG=Eastern Cooperative Oncology Group; BCLC=Barcelona Clinic for Liver Cancer; TNM=tumour, node, metastases.

4.4.3 Circulating neutrophils and platelets increase with HCC TNM stage

The mean total WCCs, neutrophils and platelets increased significantly in association with more advanced tumour stage as determined by the TNM system **Figure 4.1**. Similarly, the NLR and SII increased, although the significance of these associations were driven largely by neutrophils and platelets, as there was no clear difference in lymphocyte numbers based on TNM.

4.4.4 Circulating lymphocytes fall in association with deteriorating liver function

In contrast to tumour stage, study of circulating cells in association with the Child-Pugh grade showed no significant changes in WCC or neutrophil numbers **Figure 4.2**. On the other hand, there were significant decreases in numbers of lymphocytes and platelets with poorer liver function. The increase in the NLR was driven in this circumstance by reduced lymphocytes rather than elevated neutrophils. There was no significant difference between the SII score of the three different Child-Pugh grades in this cohort.

4.4.5 Stepwise increases in circulating neutrophils with BCLC stage, while lymphocytes fall

Total white cells increased stepwise in association with the composite BCLC staging system, **Figure 4.3**, incorporating both associations with tumour stage and liver function. The BCLC staging system also incorporates an assessment of performance status, the ECOG PST. The striking inverse associations – increases for neutrophils and decreases for lymphocytes – reflect similar changes seen in association with ECOG PST, as shown in **Figure 4.4**.

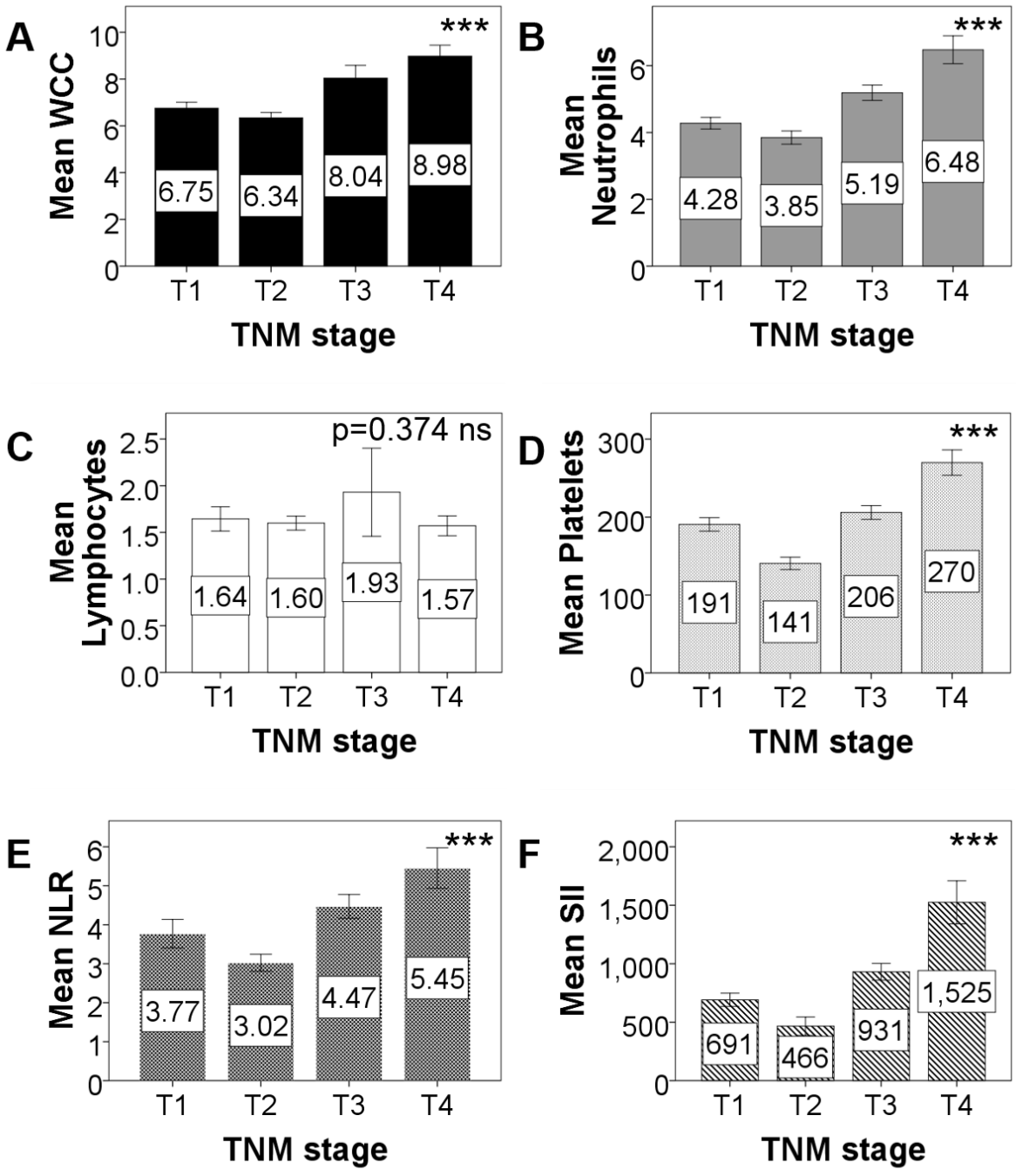


Figure 4.1 Newcastle cohort – mean number of circulating inflammatory cells across TNM stages A. Mean white cell count; B. Neutrophils; C. Lymphocytes; D. Platelets; E. NLR; F. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). WCC=white cell count.

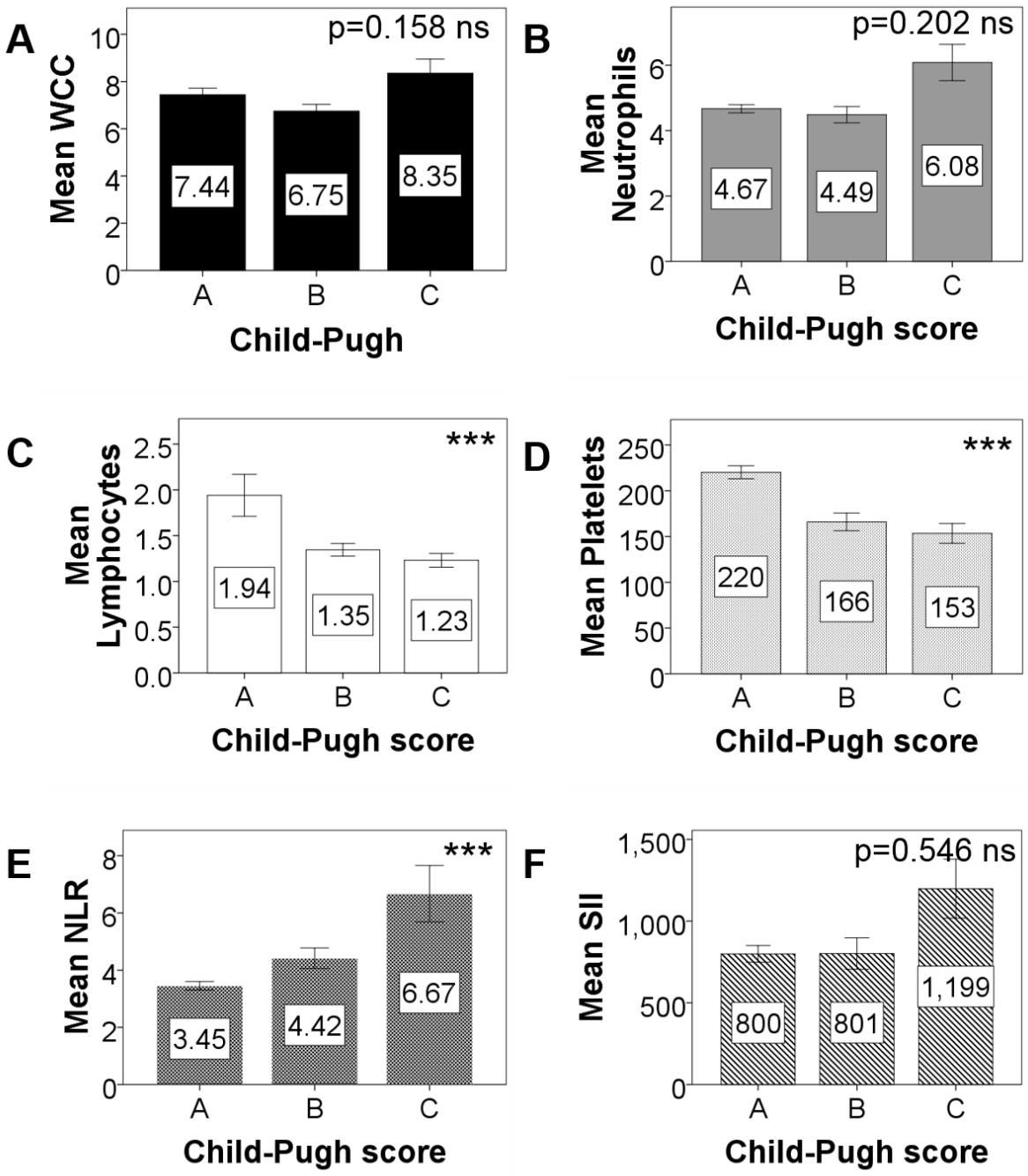


Figure 4.2 Newcastle cohort – mean number of circulating inflammatory cells across Child-Pugh grades A. Median white cell count; B. Neutrophils; C. Lymphocytes; D. Platelets; E. NLR; F. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index.

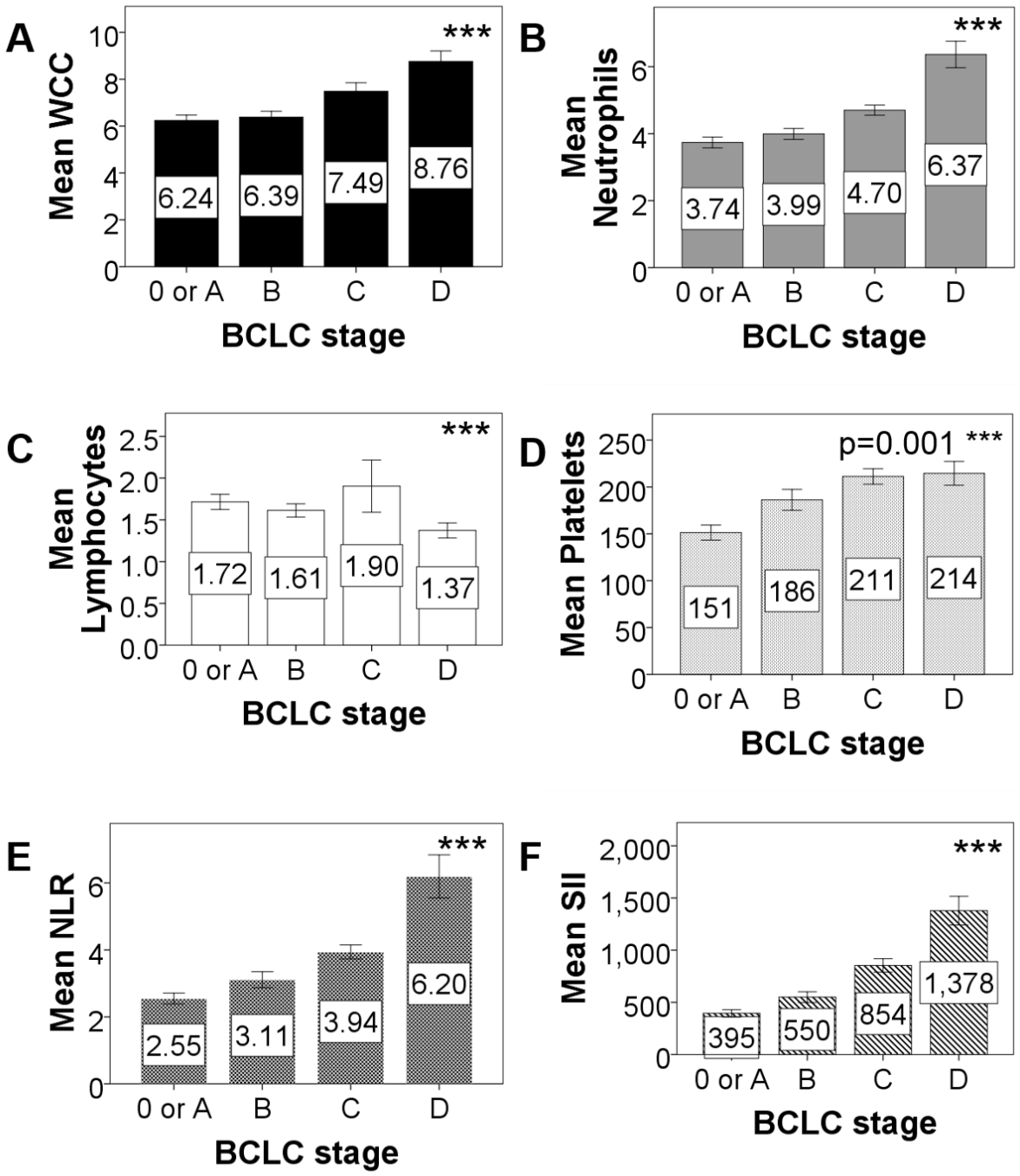


Figure 4.3 Newcastle cohort – mean number of circulating inflammatory cells across BCLC stages A. Median white cell count; B. Neutrophils; C. Lymphocytes; D. Platelets; E. NLR; F. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; BCLC=Barcelona Clinic for Liver Cancer.

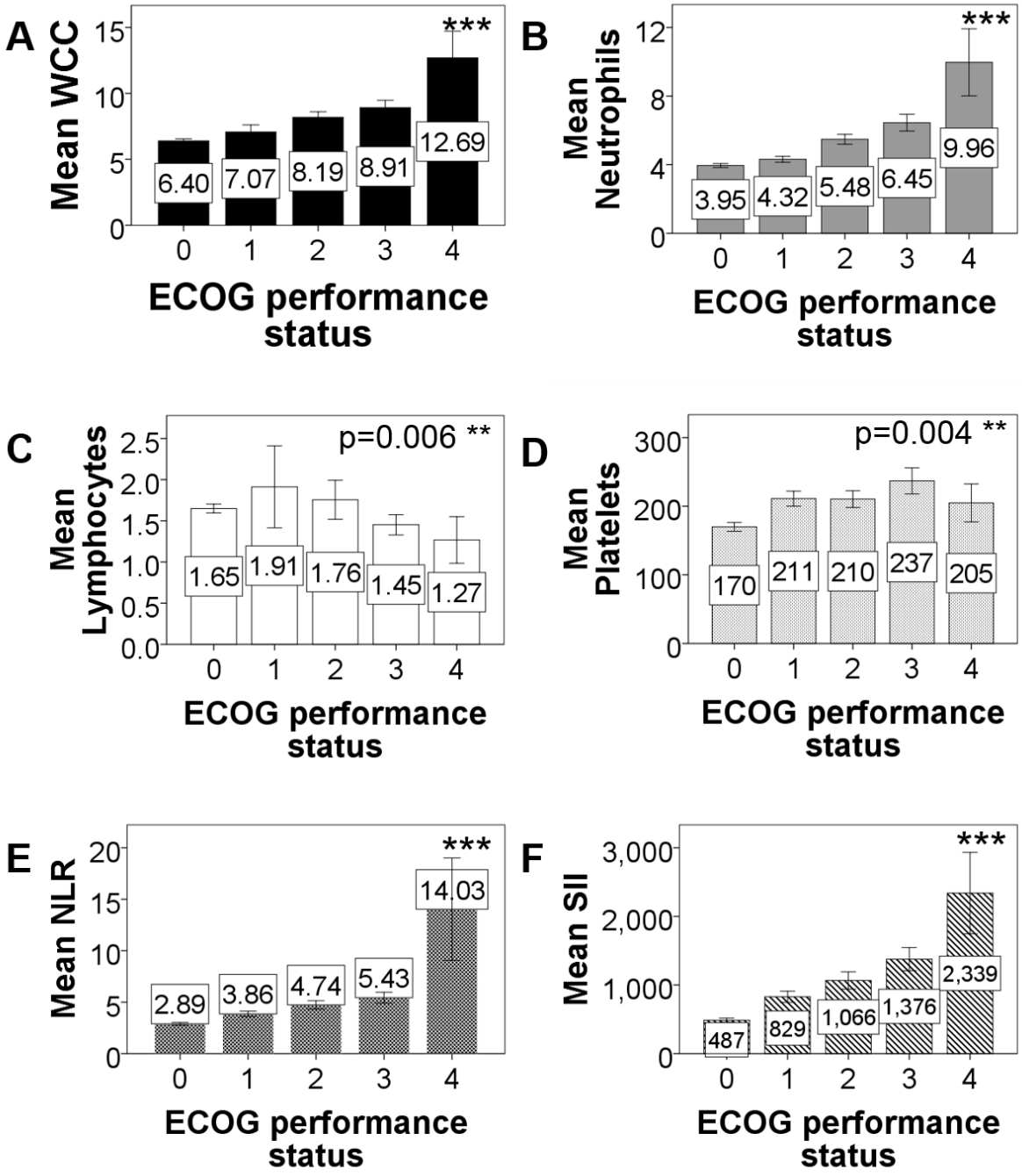


Figure 4.4 Newcastle cohort – mean number of circulating inflammatory cells and ECOG performance status A. Median white cell count; B. Neutrophils; C. Lymphocytes; D. Platelets; E. NLR; F. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; ECOG= Eastern Cooperative Oncology Group.

4.4.6 Newcastle cohort - associations with prognostic factors in patients with HCC

Exploration of bivariate associations between the combination prognostic tools, NLR and SII, performed largely as expected in this cohort **Table 4.2**, showing positive associations with poor prognostic factors (tumour size, extrahepatic disease, vascular invasion, AFP), as well as negative associations with albumin – a biomarker of preserved liver function and a better prognosis. These findings are in keeping with falling numbers of lymphocytes and/or rising numbers of neutrophils and/or platelets being associated with a poorer prognosis. The value of this large cohort, however, is the potential to study the contributions of individual cell types, with the hope of gaining a clearer understanding of the driving changes underlying the biology of progressive disease. It is clear that all cell types **Table 4.2** (neutrophils, lymphocytes and platelets) show declining numbers in association with cirrhosis, in keeping with the presence of portal hypertension and hypersplenism reducing numbers of these cells. Within that environment however, both neutrophils and platelet numbers, but not lymphocyte numbers, associated positively and highly significantly with tumour size. Data were similar for extra hepatic disease, vascular invasion, and constitutional symptoms, although in the latter two cases (vascular invasion and constitutional symptoms) there was a weak but significant negative association with lymphocyte number – in keeping with a protective association with increased circulating lymphocytes. A positive association between lymphocyte number and serum albumin was supportive of this, although may simply reflect less severe portal hypertension/hypersplenism, as there was a similar positive association with platelet number. On a similar note, rising bilirubin is a strong predictor of declining liver function and all inflammatory cells correlated negatively with this factor, perhaps highlighting the difficulties associated with understanding the immune system dysregulation in the presence of end stage liver disease and portal hypertension.

While exploring bivariate associations of staging parameters at diagnosis is interesting, the biological significance of weak bivariate associations when a number of different parameters can contribute to progression should be interpreted cautiously. Interpreting these data alongside the staging data (TNM, Child Pugh, BCLC, ECOG PST), however, suggests that advanced tumour stage is associated with rising numbers of neutrophils and platelets, rather than falling numbers of lymphocytes – the latter associated with or perhaps a consequence of

deteriorating liver function. Ideally, these studies should be supported with longitudinal assessments, such time to progression or survival, where multivariate analyses can be used to identify independent predictors of outcome.

Following Bonferroni adjustment of the p-value to correct for the number of correlations being tested, the association between vascular invasion and lymphocytes/platelets was no longer significant. The same was true for the association between lymphocytes and constitutional symptoms and the association between SII with albumin and bilirubin **Table 4.3**.

Variable	Neutrophils	Lymphocytes	Platelets	NLR	SII
Number of HCC	0.070, p=0.098	-0.048, p=0.254	-0.017, p=0.688	0.105, p=0.012*	0.070, p= 0.097
Size of HCC	0.302, p<0.001**	-0.024, p=0.573	0.432. p<0.001**	0.255, p<0.001**	0.428, p<0.001**
EHD	0.222, p<0.001**	-0.009, p=0.832	0.260, p<0.001**	0.165, p<0.001**	0.272, p<0.001**
Vascular invasion	0.171, p<0.001**	-0.106, p=0.010*	0.113, p=0.006**	0.210, p<0.001**	0.197, p<0.001**
Constitutional symptoms	0.226, p<0.001**	-0.117, p=0.005**	0.220, p<0.001**	0.253, p<0.001**	0.309, p<0.001**
Cirrhosis	-0.267, p<0.001**	-0.172, p<0.001**	-0.487, p<0.001**	-0.072, p=0.080	-0.337 p<0.001**
AFP	0.186, p<0.001**	-0.059, p=0.156	0.164, p<0.001**	0.184, p<0.001**	0.227, p<0.001**
Albumin	-0.034, p=0.416	0.179, p<0.001**	0.178, p<0.001**	-0.107, p=0.010**	0.024, p=0.561
Bilirubin	-0.147, p<0.001**	-0.252, p<0.001**	-0.397, p<0.001**	0.095, p=0.021*	-0.177, p<0.001**

Table 4.2 Newcastle cohort – bivariate associations between prognostic factors and circulating inflammatory cells. HCC=hepatocellular cancer; EHD=extra-hepatic disease; AFP=alphafetoprotein; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

Variable	Neutrophils	Lymphocytes	Platelets	NLR	SII
Number of HCC	0.070, p=0.098	-0.048, p=0.254	-0.017, p=0.688	0.105, p=0.012	0.070, p= 0.097
Size of HCC	0.302, p<0.001*	-0.024, p=0.573	0.432. p<0.001*	0.255, p<0.001*	0.428, p<0.001*
EHD	0.222, p<0.001*	-0.009, p=0.832	0.260, p<0.001*	0.165, p<0.001*	0.272, p<0.001*
Vascular invasion	0.171, p<0.001*	-0.106, p=0.010	0.113, p=0.006	0.210, p<0.001*	0.197, p<0.001*
Constitutional symptoms	0.226, p<0.001*	-0.117, p=0.005	0.220, p<0.001*	0.253, p<0.001*	0.309, p<0.001*
Cirrhosis	-0.267, p<0.001*	-0.172, p<0.001*	-0.487, p<0.001*	-0.072, p=0.080	-0.337 p<0.001*
AFP	0.186, p<0.001*	-0.059, p=0.156	0.164, p<0.001*	0.184, p<0.001*	0.227, p<0.001*
Albumin	-0.034, p=0.416	0.179, p<0.001*	0.178, p<0.001*	-0.107, p=0.010	0.024, p=0.561
Bilirubin	-0.147, p<0.001*	-0.252, p<0.001*	-0.397, p<0.001*	0.095, p=0.021	-0.177, p<0.001*

Table 4.3 Newcastle cohort – bivariate associations between prognostic factors and circulating inflammatory cells with p-value adjusted following Bonferroni correction. HCC=hepatocellular cancer; EHD=extra-hepatic disease; AFP=alphafetoprotein; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

4.4.7 Newcastle cohort – survival analyses

Univariate Cox regression analysis was performed to identify variables significantly associated with survival. Those explored included patient factors (sex, metabolic risk factors, aetiology of chronic liver disease, presence of cirrhosis), tumour factors (number of HCC, size -diameter of largest HCC, PVT, EHD), clinical assessment of liver function (ascites, encephalopathy), serum biomarkers of tumour or liver function (AFP, albumin, bilirubin, INR), other serum biochemical parameters (creatinine, sodium). Full blood count data (WCC, neutrophils, lymphocytes, platelets), the NLR and SII prognostic scores, clinical assessment of constitutional symptoms and finally, the treatment received were also explored. Data are shown in **Table 4.4A**.

Variables that demonstrated significance of <0.01 were entered into a multivariate Cox regression model, as shown in **Table 4.4B**. As the NLR and SII include single variables entered into the multivariate analysis within their component parts, distinct multivariate analyses to assess the impact of the combined scores were performed, as shown in **Table 4.4C.** Considering the inflammatory cells in the univariate survival analyses, both numbers of circulating neutrophils and platelets were significantly associated with survival, while circulating lymphocytes were not. In the multivariate study, neutrophils were independently associated with survival (p=0.015, HR 1.052, 95% CI 1.010-1.097), but platelets were not **Table 4.4B**. The SII and NLR were also significant in both univariate (p<0.001) and multivariate survival analyses (p<0.001) and associations with those key biomarkers predicting survival shown in **Table 4.2.**

A. Univariate analysis Variable	Significance
BMI	0.325
Gender	0.626
Aetiology	0.154
Cirrhosis	0.936
T2DM	0.208
Number of HCC	<0.001
Size of largest HCC	<0.001
EHD	<0.001
PVT	<0.001
Ascites	<0.001
Encephalopathy	0.371
AFP	0.013
Albumin	<0.001
Bilirubin	<0.001
INR	0.084
Sodium	0.528
Creatinine	0.549
WCC	<0.001
Neutrophils (cont.)	<0.001
Lymphocytes (cont.)	0.674
Platelets (cont.)	<0.001
Constitutional symptoms	<0.001
Treatment	<0.001
NLR	<0.001
SII	<0.001

B. Multivariate analysis Variable	Significance	HR	95% Confidence Interval	
			Lower	Upper
Number of HCC	0.022	1.040	1.006	1.076
Size of HCC	0.001	1.046	1.019	1.073
EHD	0.059	1.314	0.990	1.745
PVT	0.001	1.495	1.173	1.907
Ascites	0.092	1.247	0.965	1.612
Albumin	<0.001	0.944	0.925	0.964
Bilirubin	0.143	1.002	0.999	1.004
Neutrophils (cont.)	0.015	1.052	1.010	1.097
Platelets (cont.)	0.463	1.000	0.999	1.001
Const. symptoms	<0.001	2.089	1.664	2.621
Rx (OLTx)	<0.001			
Rx (resection)	0.228	1.526	0.768	3.032
Rx (RFA)	<0.001	4.490	2.674	7.541
Rx (HAT)	<0.001	8.034	4.932	13.088
Rx (Medical)	<0.001	6.101	2.413	15.425
Rx (BSC)	<0.001	12.74	7.769	20.888

C. Multivariate analyses - assessing the association of either NLR or SII (other parameters not shown – data very similar to above, in both analyses)				
WCC	0.867	1.002	0.982	1.022
NLR	<0.001	1.042	1.019	1.066
SII	<0.001	1.000	1.000	1.000

Table 4.4 Newcastle cohort survival analyses where all inflammatory cell data were analysed as continuous variables. A. univariate cox regression analyses. B. multivariate analysis including variables that were significant at a p-value<0.01). C. multivariate analysis assessing NLR and SII combination tools without confounding by inclusion of individual components of scores. Data for other factors entered into the multivariate analyses were as for Table 1.3B. HR= Hazard Ratio; BMI=body mass index; T2DM=type 2 diabetes mellitus; HCC=hepatocellular cancer; EHD=extra-hepatic disease; PVT=portal vein thrombus; AFP=alphafetoprotein; INR=international normalised ratio; WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index; Rx = treatment; OLTx=orthotopic liver transplant; RFA=radiofrequency ablation; HAT=hepatic arterial therapy; BSC=best supportive care.

4.4.8 Newcastle cohort – exploring clinical relevance of predictive value of circulating inflammatory cells, the NLR and the SII

While there may be value in cohort studies exploring the relationships of inflammatory cells as continuous variables with patient parameters and clinical outcomes, the clinical impact of a single circulating cell count may be small. Here we have explored the value of grouping patients categorically on the basis of whether their cell counts were above or below median value. There is the possibility that studied in large enough cohorts, these data could have value – supporting the development of biological hypotheses of progression, but also as either independent or contributory to biomarker scores (such as the NLR or SII) that have clinical utility. There may be value not just in predicting survival, but in predicting those patients who might be best served by one therapy over another – i.e. as predictive biomarkers.

The group's overall survival (OS) data, above and below the median for circulating neutrophils, lymphocytes, platelets and NLR or as three groups based on an SII <500, >500 but <2000 or >2000 are shown as Kaplan-Meier plots in **Figure 4.5.** In the Newcastle cohort, all groupings based on inflammatory cell count or ratio showed significant differences in survival times. Patients with high WCC, neutrophil, platelet, NLR and SII scores had shorter median OS (Log-Rank p<0.001) **Table 4.5** and **Figure 4.5**. A high lymphocyte count was associated with a longer median OS (Log-Rank, p<0.001) **Table 4.5** and **Figure 4.5**.

The median OS for patients with an NLR above the median for the Newcastle cohort was 6.5 months (95% CI 4.8-8.2) compared to those with an NLR below median which was 17.5 months (95% CI 14.3-20.7) **Table 4.5**. Differences in median OS were also observed when the cohort was divided into three categories based on SII score. Patients with a high SII score (>2000) had the lowest median OS - 2.0 months (95% CI 0.8-3.2) - compared to those with a medium (SII>500 but <2000) or low SII score (<500) where survival was 7.7 months (95% CI 5.1-10.3) and 19.7 months (95% CI 15.8-23.6) respectively (Log-Rank, p<0.001) **Table 4.5**.

Circulating immune cell/score	Months < median	95% CI		Months > median	95% CI		p-value
		Lower	Upper		Lower	Upper	
WCC	15.1	11.8	18.4	8.1	5.8	10.5	<0.001
Neutrophils	16.9	13.6	20.2	7.6	5.3	9.9	<0.001
Lymphocytes	8.5	5.8	11.3	14.7	11.9	17.5	<0.001
Platelets	15.6	11.9	19.3	7.2	4.7	9.7	<0.001
NLR	17.5	14.3	20.7	6.5	4.8	8.2	<0.001
SII	**Months survival**			**p-value**			
SII<500	19.7	15.8	23.6	<0.001			
SII>500<2000	7.7	5.1	10.3				
SII>2000	2.0	0.8	3.2				

Table 4.5 Newcastle cohort – Kaplan-Meier survival analysis showing median survival in months in patients with above or below median circulating inflammatory cell counts or scores (median WCC = 6.7; median neutrophil count = 4.1; median lymphocyte count = 1.4; median platelet count = 169; median NLR = 2.9; SII was divided into three categories: SII<500, SII>500 but <2000, SII>2000). Upper and lower 95% confidence intervals are shown along with Log-Rank p-value. WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index; CI=confidence interval.

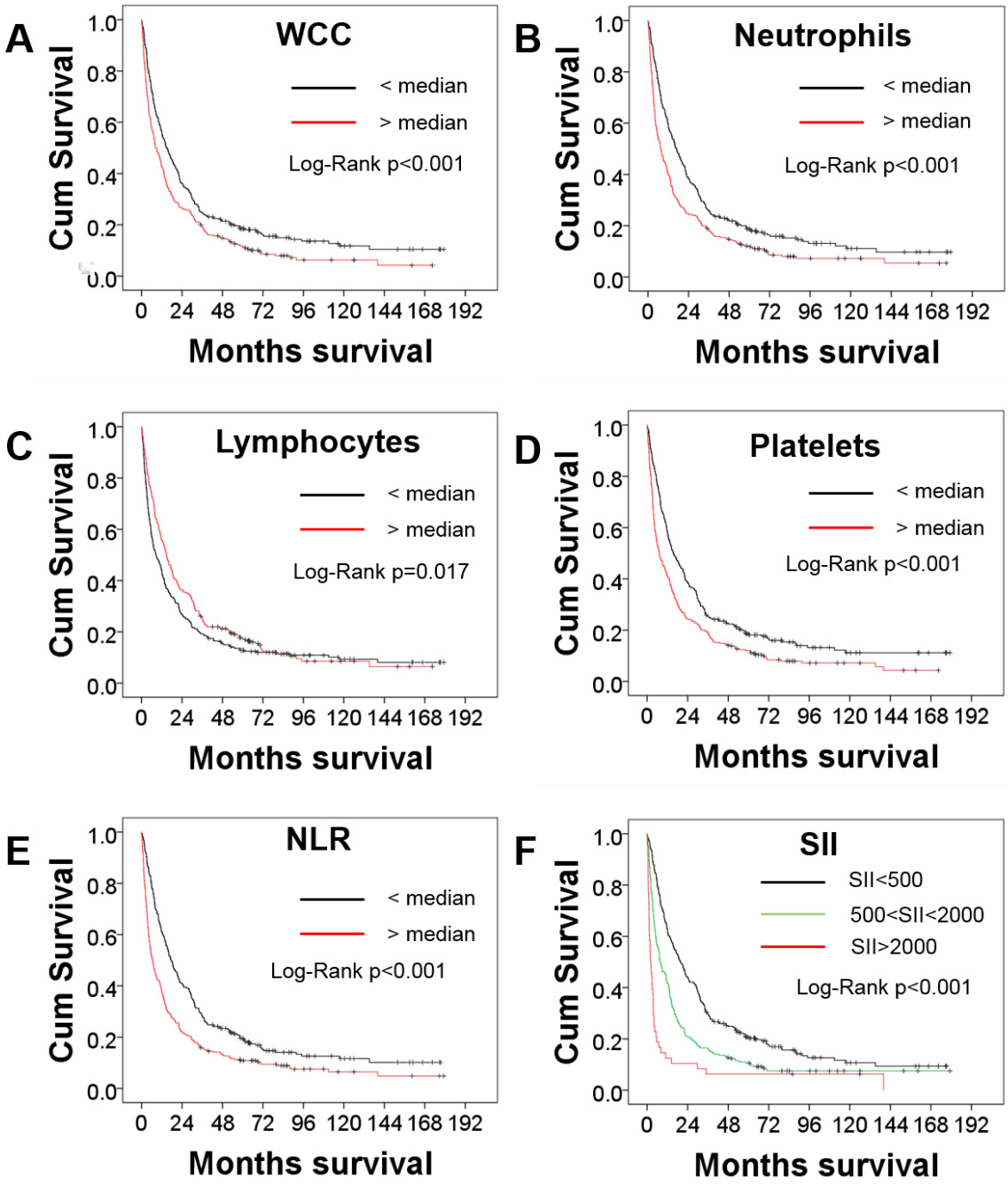


Figure 4.5 Newcastle cohort - Kaplan-Meier survival curves to show to differences between patients with above or below median A. white cell count B. neutrophil count C. lymphocyte count D. platelet count E. neutrophil to lymphocyte ratio. F. Patients were divided into three groups on the basis of having a low (<500), medium (SII>500 but <2000) or high (>2000) SII. NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

When cell counts were considered as categorical values in the multivariate analysis, neutrophils above or below the median (4.1) was independently associated with a poorer survival, with a hazard ratio of 1.33 **Table 4.6**. The NLR and SII were also independently associated with survival **Table 4.6**. SII >2000 had the greater hazard ratio (2.83), identifying a sub-group of 48 patients with an almost 3 fold greater likelihood of hazard (death) compared to patients with an SII <2000.

A. Univariate analysis Variable	p-value
WCC	<0.001
Neutrophils	<0.001
Lymphocytes	0.017
Platelets	<0.001
NLR	<0.001
SII	<0.001

B. Multivariate analysis Variable	p-value	HR	95% Confidence Interval	
			Lower	Upper
WCC	0.034	1.255	1.017	1.549
Platelets	0.977	1.003	0.800	1.259
Neutrophils	0.007	1.333	1.083	1.641
NLR	0.002	1.360	1.118	1.654
SII <500	<0.001			
SII>500 but <2000	0.035	1.258	1.017	1.556
SII>2000	<0.001	2.830	1.906	4.201

Table 4.6 Newcastle cohort - Cox regression analysis of cell counts as categorical variables were also analysed. Data were categorised into above or below median values (median WCC = 6.7; median neutrophil count = 4.1; median lymphocyte count = 1.4; median platelet count = 169; median NLR = 2.9; SII was divided into three categories: SII<500, SII>500 but <2000, SII>2000). A. Univariate analyses. Variables that were significant <0.01 were entered into a multivariate analysis. B. Multivariate analysis containing cell counts/scores as categorical variables and the variables that were significant <0.01 in univariate analyses in Table 4.4A.

These data support the roles of the combination NLR or SII scores as tools predicting prognosis in a large heterogeneous consecutive cohort of patients with HCC presenting to a single centre (Newcastle). In this cohort, however, it appeared that the key component of the tools was in fact an elevated neutrophil count, rather than elevated platelet count or falling lymphocyte count. We sought to validate this finding in a large centre in Hong Kong.

4.5 Hong Kong cohort

4.5.1 Hong Kong cohort description

As previously considered, the aetiology of HCC in Asia is largely HBV. This was reflected in the large patient cohort describing consecutive patients presenting to the Prince of Wales Hospital, Sha Tin, Hong Kong between 1st January 2007 and 31st July 2013, as shown in **Table 4.7.** As expected, HBV was the most common aetiology underlying HCC (79%). In this cohort, there was a larger proportion of males to females (88% vs 12%). The median age of patients was slightly lower than the Newcastle cohort (60 years). Patients had the presence of cirrhosis in over half of cases and a third had PVT. The mean size of the largest tumour was 8.55 mm SEM±0.21, the mean tumour number was 1.63 SEM±0.02. EHD was present in less than one quarter (~23%) of cases. In this patient cohort, patient performance status was 0-1 in 94% of patients. Most patients' (69%) underlying liver disease was classified as Child-Pugh A. More than three quarters (78%) of the cohort had HCC that was classified as BCLC B/C and using the TNM system, 58% of HCC in the cohort were deemed to be TNM stage IV. In terms of treatment, despite the majority of patients presenting with underlying liver disease classed as Child-Pugh A with good ECOG performance status, the majority had advanced diseased classed as TNM IV or BCLC C/D and the most common form of treatment reported was best supportive care (BSC).

Patient demographics			
Male/Female	516/69		
Age (median)	60		
Aetiology			
HBV **HVC** **HBV & HCV** **Other**	463 41 6 75		
Cirrhosis (Absent/Present)	246/339		
Tumour characteristics			
Number of tumours	1.63 ±0.02		
Size of largest tumour (mm)	8.55 ±0.21		
Portal vein thrombosis (Absent/Present)	390/195		
Extrahepatic disease (Absent/Present)	452/133		
Laboratory blood data			
Median AFP (median; kU/L) **White cell count (x10^9)** **Neutrophils (x10^9)** **Lymphocytes (x10^9)** **Platelets (x10^9)** **Neutrophil to lymphocyte ratio** **SII** **Albumin (g/L)** **Bilirubin (µmol/L)** **INR** **Sodium (mmol/L)** **Creatinine (µmol/L)**	265 (range 1-3,637,000) 7.01 ±0.12 4.93 ±0.11 1.31 ±0.03 206.55 ±5.51 4.94 ±0.21 1113.63 ±63.69 36.76 ±0.24 33.35 ±2.42 1.15 ±0.01 - 90.37 ±2.98		
Staging			
ECOG Performance Status		**Child-Pugh**	
0 **1** **2** **3** **4**	186 365 25 9 -	**A** **B** **C**	402 156 27
BCLC Stage		**TNM Stage**	
0/A **B** **C** **D**	94 140 316 35	**1** **2** **3** **4**	24 140 82 339
Primary Treatment			
Resection **Ablation** **Arterial treatment** **Systemic/sorafenib** **Supportive care**	68 39 129 128 221		

Table 4.7 Hong Kong cohort patient demographics (n=585). HBV=hepatitis B virus; HCV=hepatitis C virus; PBC=primary biliary cirrhosis; AIH=autoimmune hepatitis; AFP=alphafetoprotein; SII=systemic immune-inflammation index; INR=international normalised ratio; ECOG=Eastern Cooperative Oncology Group; BCLC=Barcelona Clinic for Liver Cancer; TNM=tumour, node metastases.

4.5.2 Increasing tumour stage, more extensive underlying liver disease and performance status was associated with increasing neutrophils and inflammatory scores as well as decreasing lymphocyte counts

As TNM stage, Child-Pugh scores and BCLC classification increased, so did the WCC, circulating neutrophils and both of the inflammatory scores – NLR and SII **Figure 4.6-Figure 4.9**. In all cases, there were highly statistically significant differences between the groups; tested using the Kruskal-Wallis test ($p<0.001$). Platelet counts also increased with increasing TNM stage and BCLC classification **Figure 4.6** and **Figure 4.8**; however the relationship between platelets and Child-Pugh score was less well-defined with little difference seen between the groups **Figure 4.7**. Platelets increased with ECOG performance status 0-2 in a stepwise manner but although there were significant differences between the groups, platelet counts fell in patients with PST 3 **Figure 4.9**. Conversely, the number of lymphocytes decreased with increased TNM stage ($p=0.020$); worsening Child-Pugh ($p=0.009$); BCLC ($p<0.001$) and ECOG performance status ($p=0.001$) **Figure 4.6-Figure 4.9**.

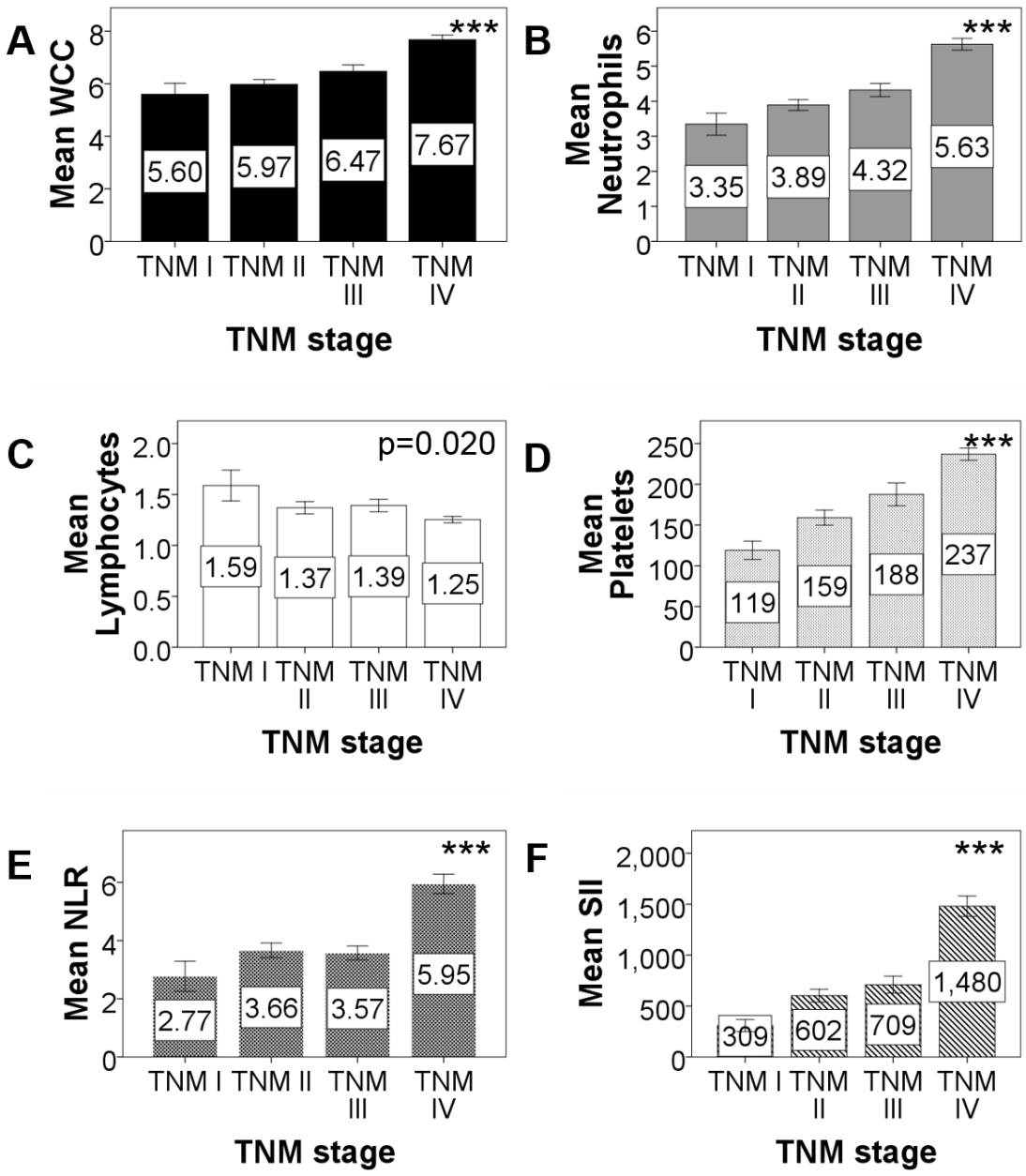


Figure 4.6 Hong Kong cohort – mean numbers of circulating inflammatory cells across TNM stages A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; TNM=tumour, node, metastases.

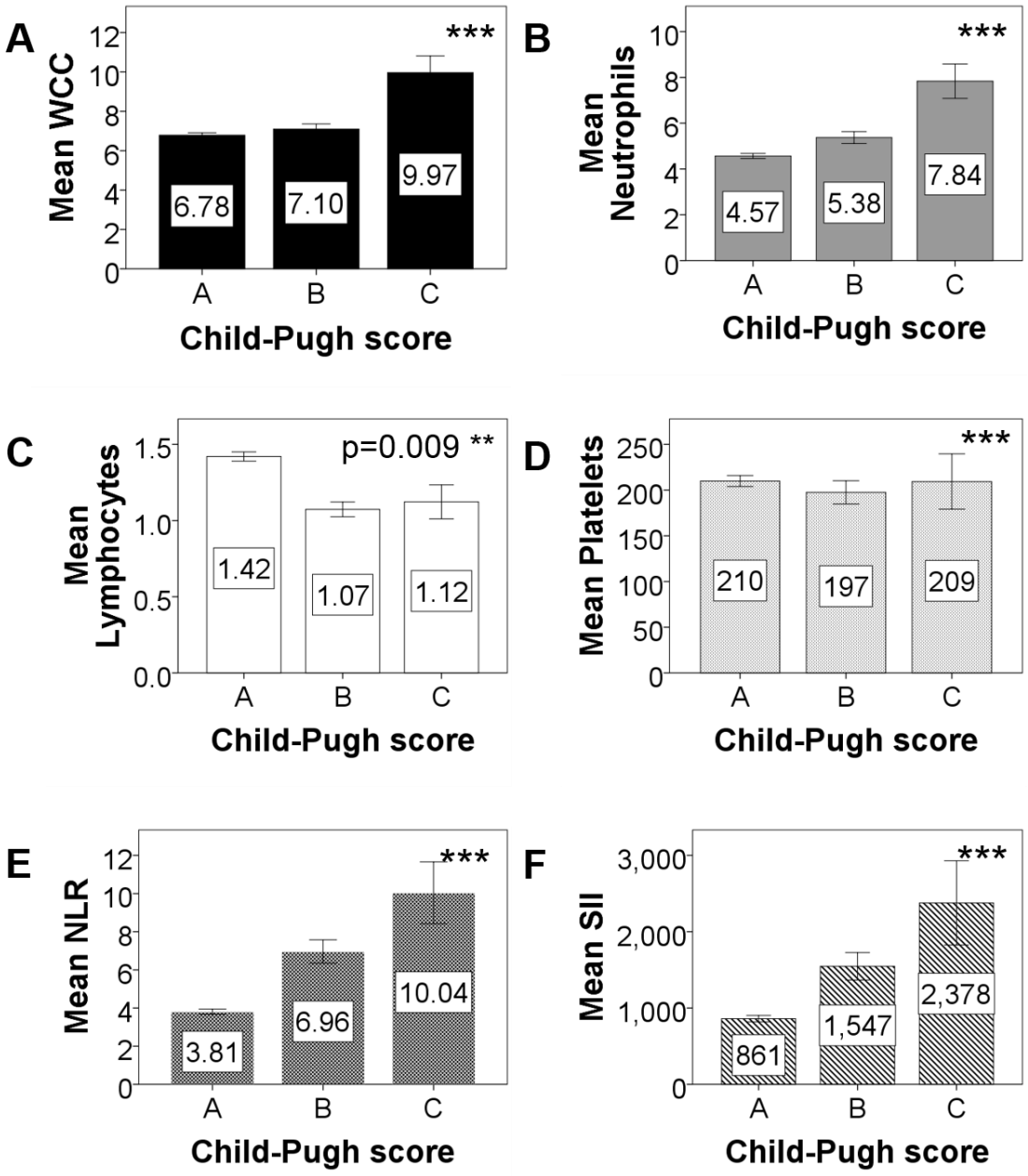


Figure 4.7 Hong Kong cohort – mean numbers of circulating inflammatory cells across Child-Pugh scores A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index.

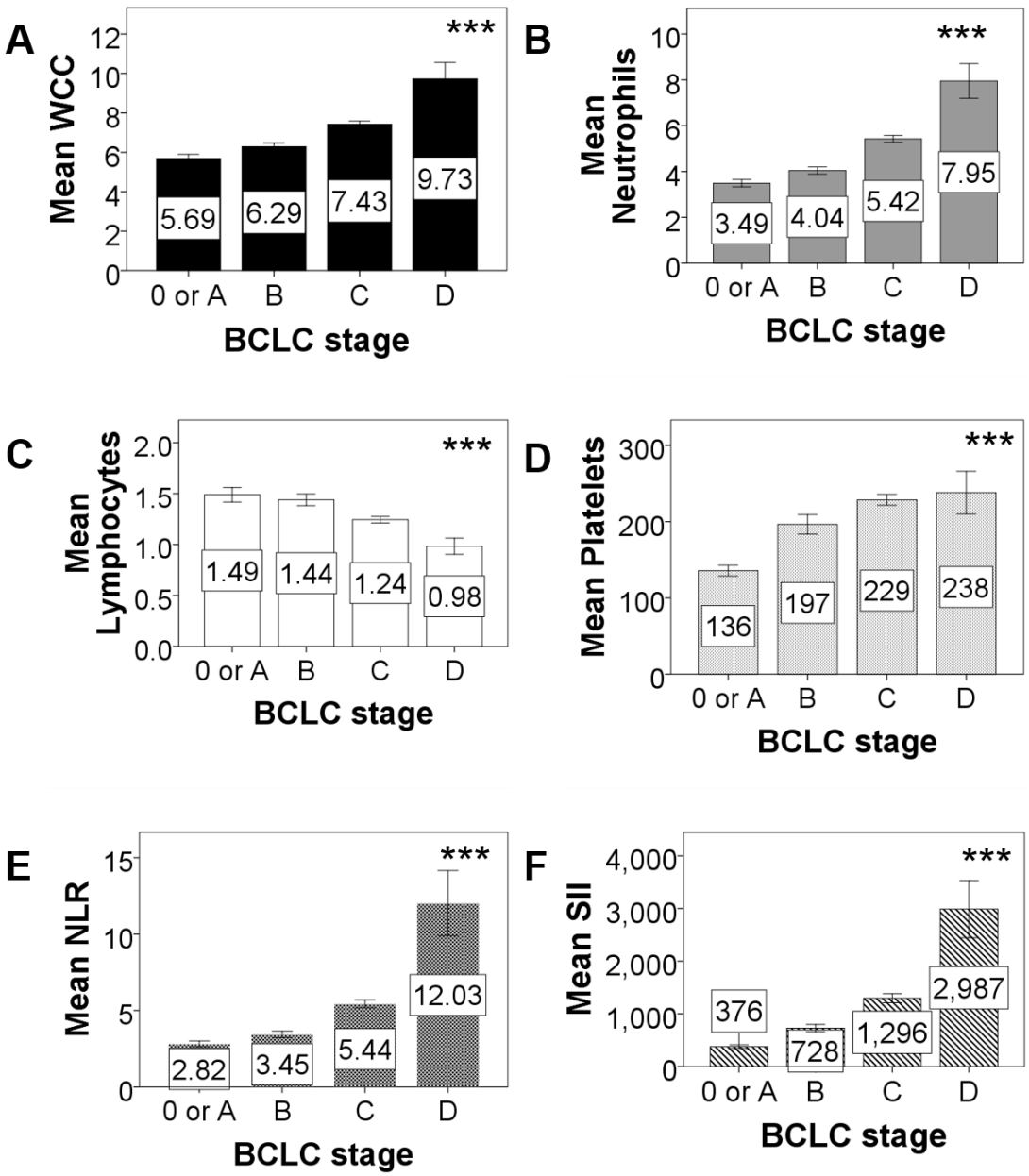


Figure 4.8 Hong Kong cohort – mean numbers of circulating inflammatory cells across BCLC stages. A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; BCLC=Barcelona Clinic for Liver Cancer.

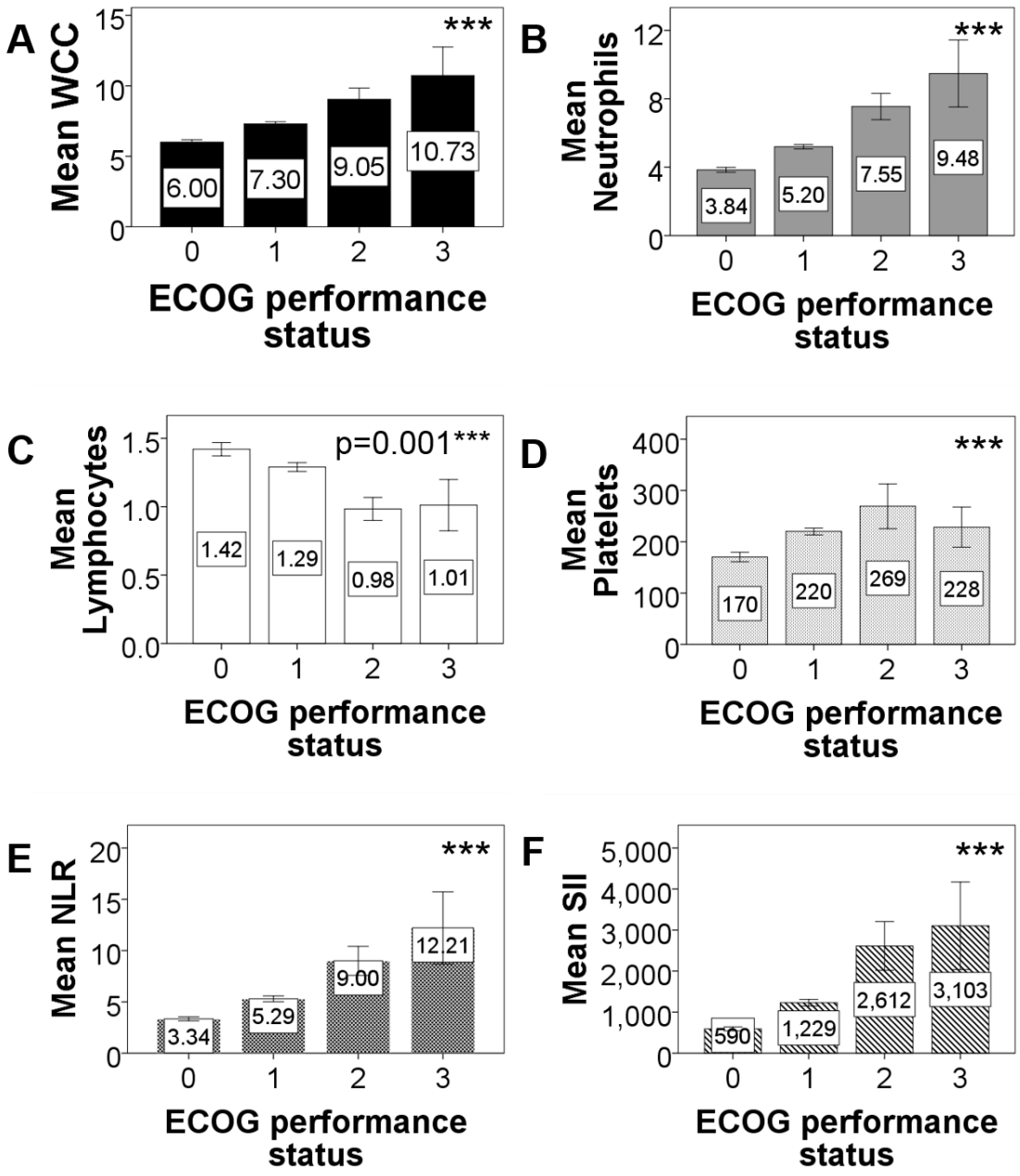


Figure 4.9 Hong Kong cohort – mean circulating inflammatory cell counts and ECOG performance status. A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; ECOG= Eastern Cooperative Oncology Group

4.5.3 Hong-Kong cohort - associations with HCC prognostic factors

Bivariate correlations observed in the Hong-Kong cohort were similar to those observed in the Newcastle cohort. Positive correlations were observed between neutrophils, platelets, the NLR, SII with tumour number and diameter, EHD, vascular invasion, constitutional symptoms AFP and bilirubin **Table 4.8**. Negative correlations were observed between neutrophils, the NLR and SII with cirrhosis and albumin **Table 4.8**. Lymphocytes correlated inversely with tumour size, vascular invasion, constitutional symptoms, AFP and bilirubin **Table 4.8**. There was a positive association between lymphocytes and serum albumin **Table 4.8**.

As for the Newcastle cohort, when the Bonferroni correction was applied to account for multiple associations, the association between lymphocytes and constitutional symptoms was no longer significant. Also, Bonferroni adjustment of the p-value meant to the associations between neutrophils and serum bilirubin; and the NLR and cirrhosis were no longer statistically significant **Table 4.9**.

Variable	Neutrophils	Lymphocytes	Platelets	NLR	SII
Number of HCC	0.176, p<0.001**	-0.022, p=0.600	0.154, p<0.001**	0.145, p<0.001**	0.200, p<0.001**
Size of HCC	0.358, p<0.001**	-0.141, p<0.001**	0.453, p<0.001**	0.382, p<0.001**	0.526, p<0.001**
EHD	0.259, p<0.001**	-0.079, p=0.057	0.197, p<0.001**	0.258, p<0.001**	0.293, p<0.001**
Vascular invasion	0.268, p<0.001**	-0.142, p=0.001**	0.169, p<0.001**	0.305, p<0.001**	0.300, p<0.001**
Constitutional symptoms	0.287, p<0.001**	-0.118, p=0.004**	0.253, p<0.001**	0.301, p<0.001**	0.349, p<0.001**
Cirrhosis	-0.215, p<0.001**	-0.068, p=0.098	-0.368, p<0.001**	-0.108, p=0.009**	-0.278, p<0.001**
AFP	0.259, p<0.001**	-0.156, p<0.001**	0.221, p<0.001**	0.309, p<0.001**	0.342, p<0.001**
Albumin	-0.176, p<0.001**	0.284, p<0.001**	0.067, p=0.104	-0.310, p<0.001**	-0.189, p<0.001**
Bilirubin	0.088, p=0.034*	-0.314, p<0.001**	-0.222, p<0.001**	0.271, p<0.001**	0.061, p=0.143

Table 4.8 Hong Kong cohort – bivariate associations between significant factors affecting survival and circulating inflammation cells. HCC=hepatocellular cancer; EHD=extra-hepatic disease; AFP=alphafetoprotein; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

Variable	Neutrophils	Lymphocytes	Platelets	NLR	SII
Number of HCC	0.176, p<0.001*	-0.022, p=0.600	0.154, p<0.001*	0.145, p<0.001*	0.200, p<0.001*
Size of HCC	0.358, p<0.001*	-0.141, p<0.001*	0.453, p<0.001*	0.382, p<0.001*	0.526, p<0.001*
EHD	0.259, p<0.001*	-0.079, p=0.057	0.197, p<0.001*	0.258, p<0.001*	0.293, p<0.001*
Vascular invasion	0.268, p<0.001*	-0.142, p=0.001*	0.169, p<0.001*	0.305, p<0.001*	0.300, p<0.001*
Constitutional symptoms	0.287, p<0.001*	-0.118, p=0.004	0.253, p<0.001*	0.301, p<0.001*	0.349, p<0.001*
Cirrhosis	-0.215, p<0.001*	-0.068, p=0.098	-0.368, p<0.001*	-0.108, p=0.009	-0.278, p<0.001*
AFP	0.259, p<0.001**	-0.156, p<0.001*	0.221, p<0.001*	0.309, p<0.001*	0.342, p<0.001*
Albumin	-0.176, p<0.001*	0.284, p<0.001*	0.067, p=0.104	-0.310, p<0.001*	-0.189, p<0.001*
Bilirubin	0.088, p=0.034	-0.314, p<0.001*	-0.222, p<0.001*	0.271, p<0.001*	0.061, p=0.143

Table 4.9 Hong Kong cohort – bivariate associations between significant factors affecting survival and circulating inflammation cells with Bonferroni adjusted p-value. HCC=hepatocellular cancer; EHD=extra-hepatic disease; AFP=alphafetoprotein; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

4.5.4 Hong Kong cohort – survival analysis

The impact of circulating immune cell counts and scores were investigated using survival analyses as previosuly described in **Section 4.4.6**. Variables that were found to be significant in univariate analyses **Table 4.10A** were entered into multivariate analyses; with separate analyses carried out for individual immune cell counts and inflammatory scores**.** In this cohort, variables that remained siginificant in multivariate analyses included PVT, AFP, treatment and neutrophils **Table 4.10B-C**.

Additionally, the follwing also remained statistically significant in a multivariate analysis: tumour number (p=0.001); tumour size (p=0.002); EHD (p=0.027); bilirubuin (p=0.001); INR (p=0.002) and constitutional symptoms (p=0.017) **Table 4.10B-C**. The NLR and SII did not remain independent prognostic indicators in the multivariate analysis **Table 4.10C**.

A. Univariate analysis Variable	Signifi -cance
Gender	0.311
Aetiology	0.354
Cirrhosis	0.083
Number of HCC	0.003
Size of largest HCC	<0.001
EHD	<0.001
PVT	<0.001
Ascites	<0.001
AFP	<0.001
Albumin	<0.001
Bilirubin	<0.001
INR	<0.001
Creatinine	0.565
Neutrophils (cont.)	<0.001
Lymphocytes (cont.)	<0.001
Platelets (cont.)	0.005
Constitutional symptoms	<0.001
Treatment	<0.001
NLR	<0.001
SII	<0.001

B. Multivariate analysis Variable	Signifi -cance	HR	95% Confidence Interval	
			Lower	Upper
Number of tumours	0.001	1.460	1.174	1.815
Size of largest tumour	0.002	1.035	1.012	1.057
EHD	0.027	1.326	1.033	1.701
PVT	<0.001	2.120	1.680	2.676
Ascites	0.688	1.052	0.822	1.345
AFP	<0.001	1.000	1.000	1.000
Albumin	0.364	0.991	0.971	1.011
Bilirubin	0.001	1.003	1.001	1.005
INR	0.002	2.487	1.400	4.416
Neutrophils	<0.001	1.113	1.073	1.156
Lymphocytes	0.125	0.873	0.735	1.038
Platelets	0.133	0.999	0.999	1.000
Constitutional symptoms	0.017	1.362	1.058	1.754
Rx (resection)	<0.001			
Rx (RFA)	0.903	1.039	0.564	1.914
Rx (HAT)	0.324	1.284	0.781	2.112
Rx (Medical)	0.042	1.700	1.020	2.835
Rx (palliative)	<0.001	2.767	1.685	4.543

C. Multivariate analyses - assessing the association of either NLR or SII				
NLR	0.208	1.009	0.995	1.022
SII	0.097	1.000	1.000	1.000

Table 4.10 Hong Kong cohort –Cox regression survival analyses A. Univariate analysis. B. Multivariate analysis. Variables that were significant (p<0.01) in a univariate analysis were entered into a multivariate analysis to determine independent variables affecting survival. HR=Hazard Ratio; HCC=hepatocellular cancer; EHD=extra-hepatic disease; PVT=portal vein thrombus; AFP=alphafetoprotein; INR=international normalised ratio; WCC=white cell count; Cont.=continuous; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index; Rx = treatment; OLTx=orthotopic liver transplant; RFA=radiofrequency ablation; HAT=hepatic arterial therapy; BSC=best supportive care.

4.5.5 Prognostic implications of circulating neutrophil counts and inflammatory scores

The prognostic implications of circulating immune cell counts and scores were further investigated with results indicating that patients with above median counts of neutrophils, platelets, NLR; SII; and below median counts of lymphocytes had a shorter median survival and these difference were very highly statistically significant (Log-Rank, p<0.001) **Table 4.11** and **Figure 4.10**. When circulating cell counts and scores were considered as categorical variables - grouped as above/below median for the cohort – neutrophils (p<0.001), the NLR (p=0.001) and SII (p<0.001) remained significant independent variables in separate multivariate analyses **Table 4.12** containing the variables that were significant in previous univariate analyses **Table 4.10**. Platelets as a categorical varibale did not remain and independent prognostic indicator in multivariate analysis **Table 4.12**. In this cohort lymphocytes were also a significant independent prognstic indicator (p=0.017) **Table 4.14**.

Circulating immune cell/score	**Months < median**	**95% CI**		**Months > median**	**95% CI**		**p-value**
		Lower	**Upper**		**Lower**	**Upper**	
Neutrophils	10.2	8.3	12.1	2.8	2.1	3.4	<0.001
Lymphocytes	3.8	2.9	4.7	8.2	6.0	10.4	<0.001
Platelets	8.2	6.2	10.2	4.2	3.4	5.0	<0.001
NLR	11.4	8.6	14.1	2.8	2.2	3.3	<0.001
SII	**Months survival**			**p-value**			
SII<500	11.9	9.0	14.8	<0.001			
SII>500 but <2000	4.6	3.8	5.5				
SII>2000	1.8	1.2	2.4				

Table 4.11 Hong Kong cohort – Kaplan-Meier survival analysis showing differences in median survival in months in patients with above or below median circulating inflammatory cell counts or scores. Median neutrophil count = 4.3; median lymphocyte count = 1.2; median platelet count = 176; median NLR = 3.5; SII was divided into three categories: SII<500, SII>500 but <2000, SII>2000. Upper and lower 95% confidence intervals are shown along with Log-Rank p-values. NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index; CI=confidence interval.

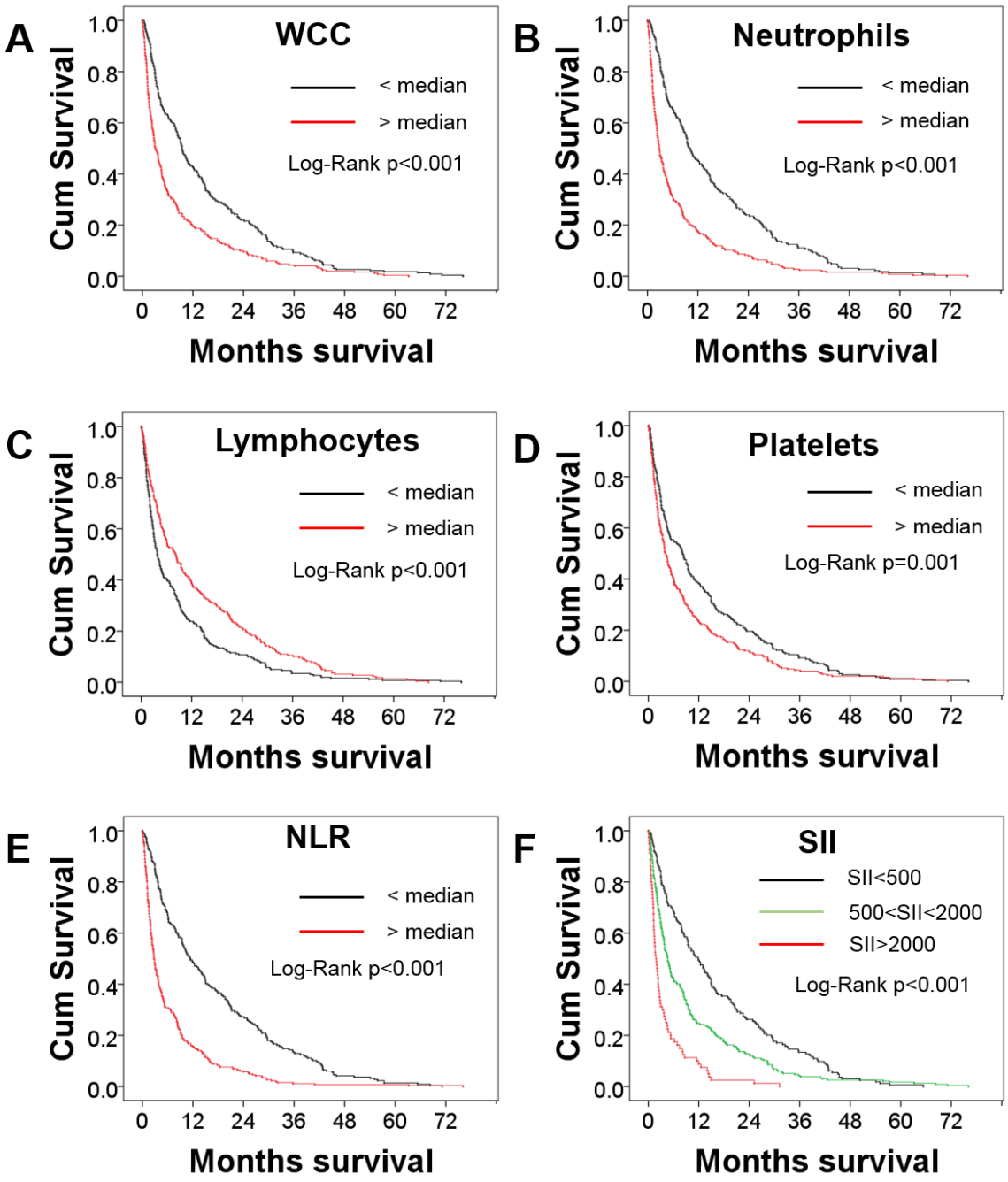


Figure 4.10 Hong Kong cohort - Kaplan-Meier survival curves to show to differences between patients with above or below median A. neutrophil count B. lymphocyte count C. platelet count D. neutrophil to lymphocyte ratio. E. Patients were divided into three groups on the basis of having a low (<500), medium (SII>500 but <2000) or high (>2000) SII. NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

A. Univariate analysis Variable	p-value
Neutrophils (cat.)	<0.01
Lymphocytes (cat.)	<0.001
Platelets (cat.)	0.001
NLR (cat.)	<0.001
SII (cat.)	<0.001

B. Multivariate analysis Variable	p-value	HR	95% Confidence Interval	
			Lower	Upper
Neutrophils (cat.)	<0.001	1.551	1.253	1.918
Lymphocytes (cat.)	0.017	0.787	0.647	0.957
Platelets (cat.)	0.793	0.970	0.770	1.221
NLR (cat.)	<0.001	1.683	1.373	2.064
SII <500	0.001			
SII>500 but <2000	0.332	1.120	0.891	1.410
SII>2000	<0.001	1.787	1.295	2.466

Table 4.12 Hong Kong cohort – Cox regression analysis A Univariate analysis. B. Multivariate analysis. Cell counts as categorical variables were also analysed. Data were categorised into above or below median values (median neutrophil count = 4.3; median lymphocyte count = 1.2; median platelet count = 176; median NLR = 3.5; SII was divided into three categories: SII<500, SII>500 but <2000, SII>2000. All categorical cell counts were significant <0.01 in univariate analyses and were entered into a multivariate analysis containing the variables that were significant in the univariate analyses in Table 4.10. In the multivariate analysis neutrophils, NLR and SII remained significant independent variable.

4.6 Combined cohort

4.6.1 Combined cohort description

In order to assess the utility of circulating immune cell counts and inflammatory scores in a larger patient cohort, the Newcastle and Hong Kong cohorts were combined. This resulted in a patient cohort of 1168 patients **Table 4.13**. The proportion of male:female was 85% vs 15%. One of the advanatges of combining the two cohorts was to increase the sample size of patients with varying aetiology and undergoing different treatment types. In the combined cohort, the most common aetiology was HBV (42%) with ALD (14%), NAFLD (11%) and HCV (9%) being the next most common aetiologies. The mean number of tumours was 2.08 SEM±0.05 and the size of the largest tumour was 7.40 SEM±0.15. PVT was reported in 30% of cases and 19% of patients had the presence of EHD. Most of the patients in the cohort (79%) were classified using ECOG as PST 0-1. 66% of assessment of patients' underlying liver disease was scored as Child-Pugh A. Using the BCLC classification, 50% had BCLC stage C compared to BCLC stage A/B or D. Overall, patients were most often staged as TNM IV (37%). The most commonly regimented treatments were loco-regional and supportive care.

Patient demographics			
Male/Female	989/179		
Age (median)	64		
Aetiology			
None/unknown	114		
Alcohol related	168		
Non-alcoholic fatty liver	127		
HBV	491		
HCV	101		
HBV & HCV	6		
PBC	19		
AIH	7		
Haemochromatosis	31		
Cryptogenic	24		
Other	80		
Cirrhosis (Absent/Present)	403/765		
Tumour characteristics			
Number of tumours	2.08 ±0.05		
Size of largest tumour (mm)	7.40 ±0.15		
Portal vein thrombosis (Absent/Present)	820/348		
Extrahepatic disease (Absent/Present)	941/227		
Laboratory blood data			
Median AFP (median; kU/L)	87.0		
White cell count (x10^9)	7.21 ±0.12		
Neutrophils (x10^9)	4.89 ±0.08		
Lymphocytes (x10^9)	1.51 ±0.07		
Platelets (x10^9)	202.72 ±3.83		
Neutrophil to lymphocyte ratio	4.53 ±0.14		
SII	984.59 ±89.57		
Albumin (g/L)	36.23 ±0.17		
Bilirubin (µmol/L)	31.25 ±1.46		
INR	1.12 ±0.01		
Sodium (mmol/L)	138.90 ±2.07		
Creatinine (µmol/L)	96.48 ±1.73		
Staging			
ECOG Performance Status		**Child-Pugh**	
0	403	**A**	771
1	525	**B**	288
2	140	**C**	107
3	87		
4	13		
BCLC Stage		**TNM Stage**	
0/A	182	**1**	238
B	234	**2**	246
C	584	**3**	250
D	168	**4**	434
Primary Treatment			
Liver transplant	53		
Resection	99		
Ablation	104		
Arterial treatment	317		
Systemic medical	137		
Best supportive care/ no treatment	458		

Table 4.13 Combined cohort patient demographics n=1491. HBV=hepatitis B virus; HCV=hepatitis C virus; PBC=primary biliary cirrhosis; AIH=autoimmune hepatitis; AFP=alphafetoprotein; SII=systemic immune-inflammation index; INR=international normalised ratio; ECOG=Eastern Cooperative Oncology Group; BCLC=Barcelona Clinic for Liver Cancer; TNM=tumour, node metastases.

4.6.2 Combined cohort- inflammatory cell counts and inflammation scores increased with increasing tumour stage and performace status

Inflammatory cell counts and inflammatory scores showed a general increase as TNM stage, BCLC classification and ECOG performance status increased, except for lymphocytes where mean counts where variable across the categories **Figure 4.11, Figure 4.12, Figure 4.13** and **Figure 4.14**. In all cases, patients with HCC classified as TNM I had higher counts of all inflammation cells and inflammatory scores than patients with TNM II after which there was a gradual increase in neutrophils, platelets, NLR and SII **Figure 4.11**.

4.6.3 Decreasing numbers of lymphocytes and platelets were associated with declining liver function

An increase in neutrophils, NLR and SII was observed as liver function declined **Figure 4.12**. Interestingly, there was a significant reduction in the number of lymphocytes and platelets **Figure 4.12.** The combination of increasing neutrophils and falling lymphocytes have led to increased NLR being significantly associated with worsening Child-Pugh. Interestingly, although the SII also increase with worsening liver function, the differences between the groups were not statistically significant: indicating that the fall in lymphocytes was more indicative of deteriorating liver function since the SII calculation places more emphasis on increase in neutrophils.

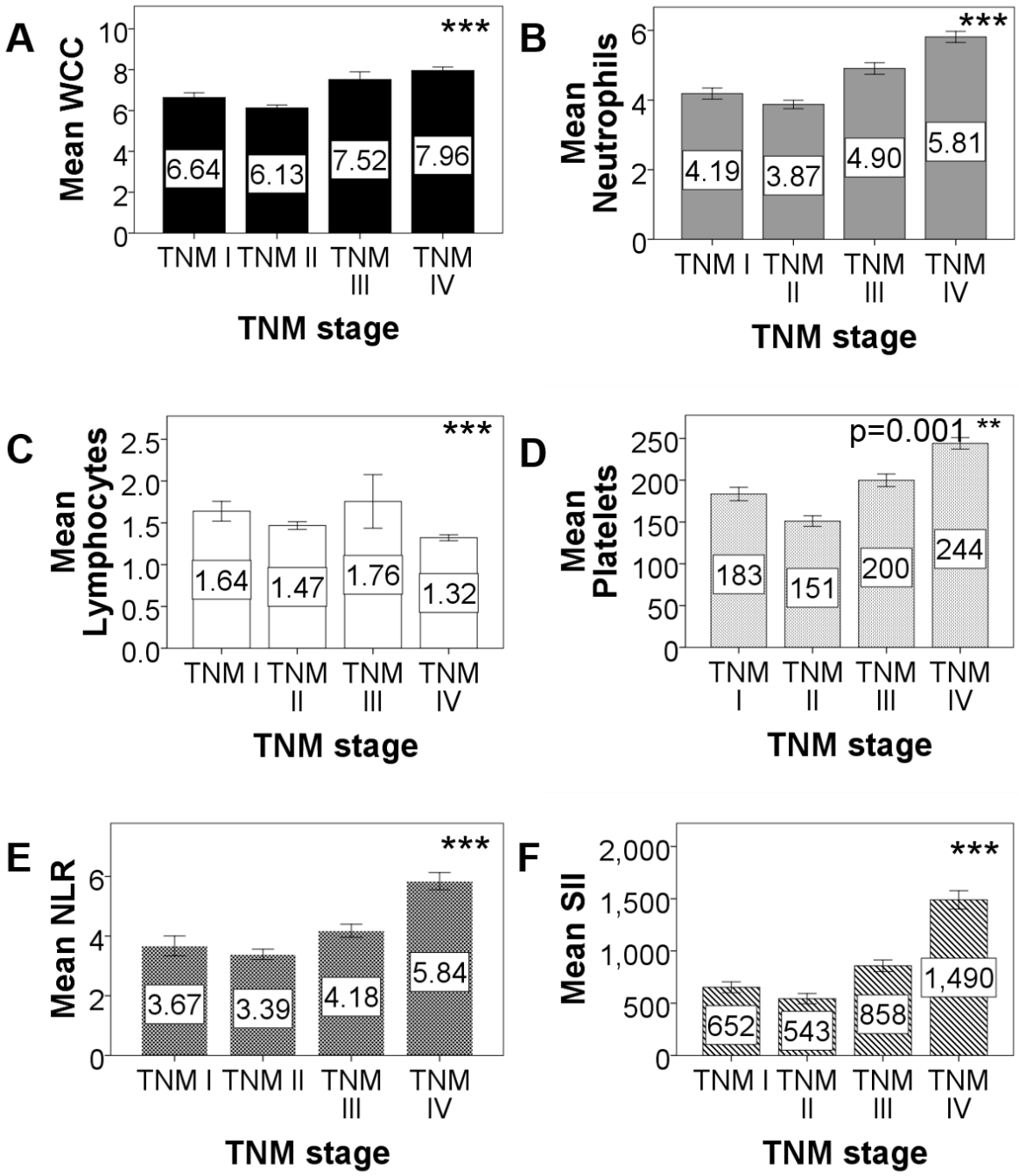


Figure 4.11 Combined cohort - - mean number of circulating inflammatory cells across TNM stages A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; TNM=tumour, node, metastases.

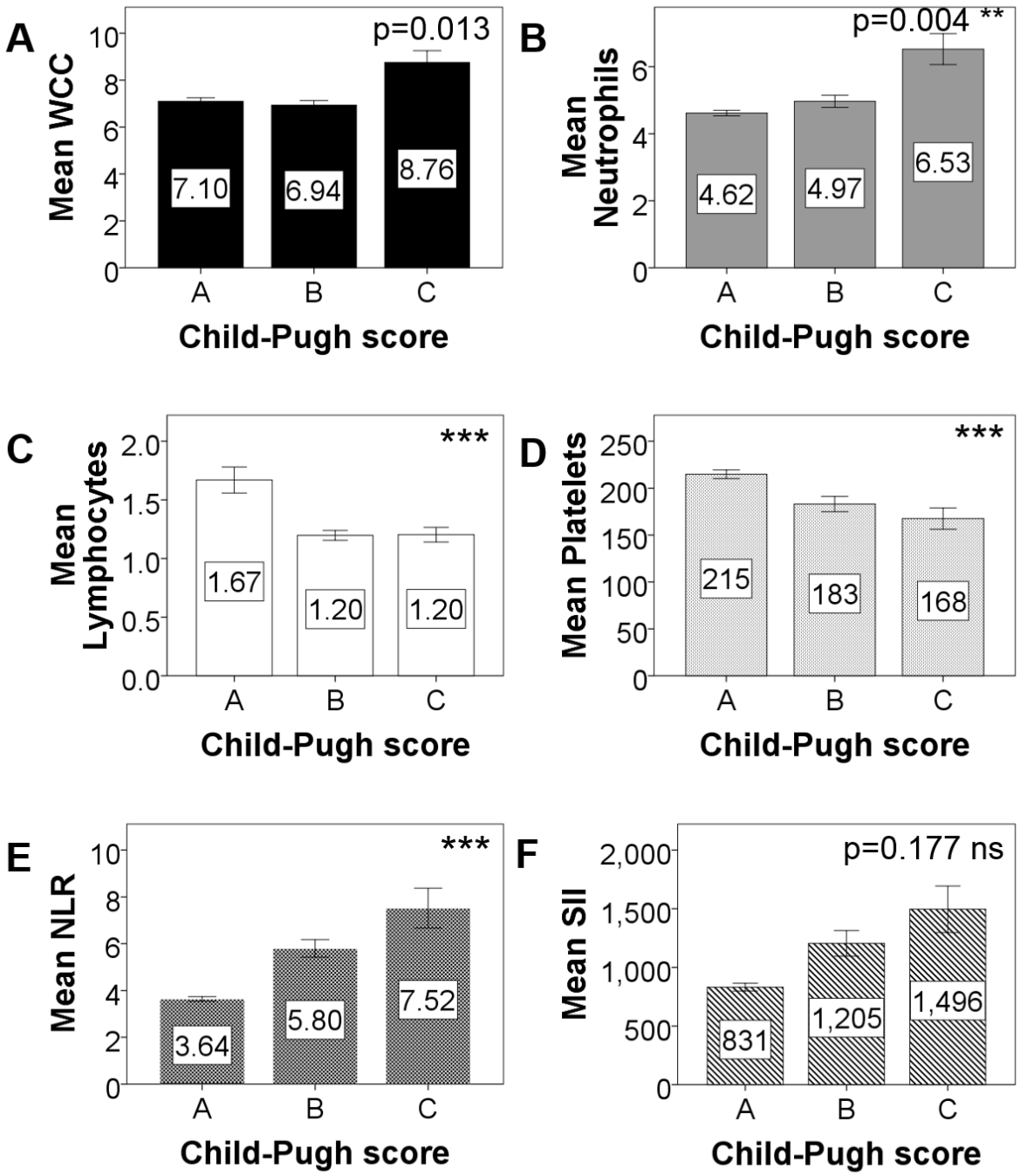


Figure 4.12 Combined cohort – mean number of circulating inflammatory cells and Child-Pugh grade A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index.

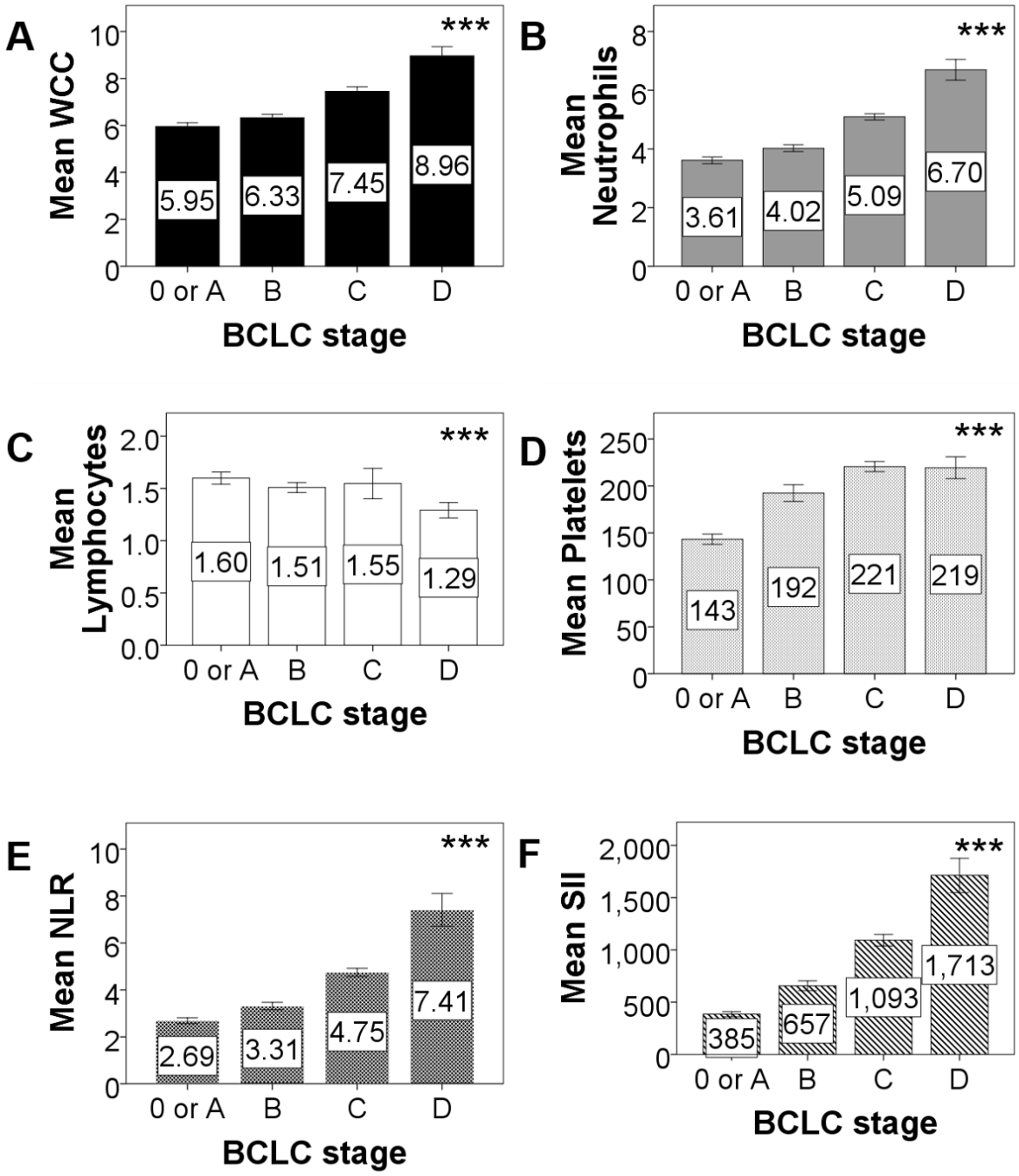


Figure 4.13 Combined cohort – mean number of circulating inflammatory cells across BCLC stages A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0.01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; BCLC=Barcelona Clinic for Liver Cancer.

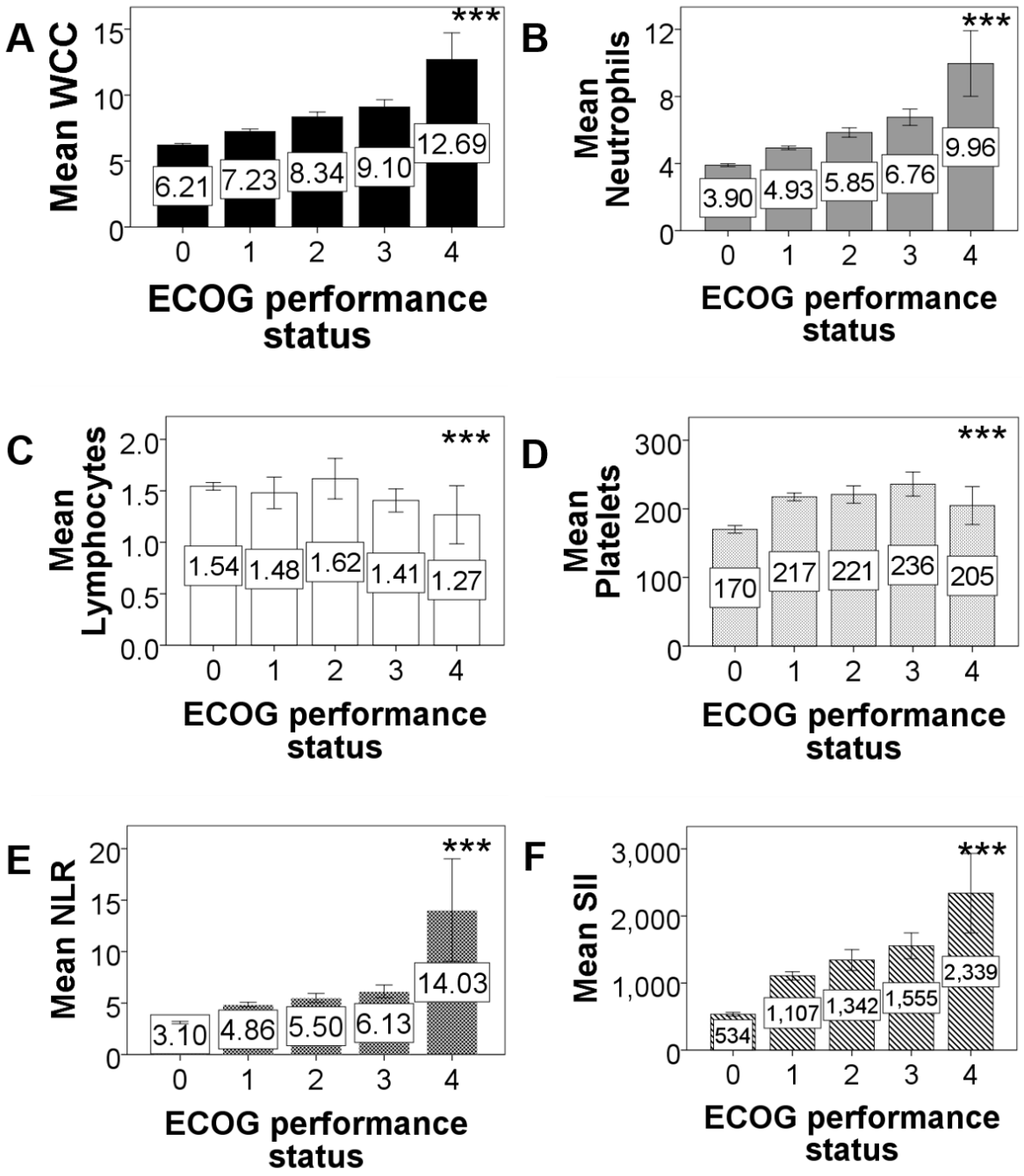


Figure 4.14 Combined cohort – mean number of circulating inflammatory cells and ECOG performance status score. A. Neutrophils; B. Lymphocytes; C. Platelets; D. NLR; E. SII. Kruskal-Wallis test (ns = not significant; *p<0.05, **p<0. 01, ***p<0.001). NLR=neutrophil to lymphocyte ratio; SII= systemic immune-inflammation index; ECOG= Eastern Cooperative Oncology Group.

4.6.4 Combined cohort – associations with HCC prognostic factors

In the combined cohort, expectedly bivariate correlations mirror the trends that were observed in the individual cohorts. On the whole, correlations between circulating cell counts and tumour characteristics or serum markers of liver function were mostly positive **Table 4.14**. Significant inverse correlations were seen between all circulating cell counts/scores and cirrhosis **Table 4.14**. Also, lymphocytes negatively correlated with all tumour features and serum markers **Table 4.14**. With the odd exception, correlations between circulating cell counts/ scores with serum albumin/bilirubin now reach statistical significance with bilirubin correlating negatively with all cell counts/ scores except NLR and albumin correlating negatively with all cell counts/ scores except lymphocytes and platelets **Table 4.14**. In this combined cohort, where the number of cases gave additional statistical power and p values for even modest associations were statistically significant, the strength of the associations can help when considering which are likely to have biological significance or clinical relevance. The strongest associations, all with p values <0.001, are highlighted in yellow. Again it is clear that poor prognostic tumour characteristics associate with increasing neutrophil and platelet number, while higher lymphocytes correlate with better liver function (higher albumin and lower bilirubin). Following Bonferroni adjustment of the p-value, the only associations that did not remain significant at the 0.001 level were those between albumin and neutrophils/SII **Table 4.15**.

Variable	Neutrophils	Lymphocytes	Platelets	NLR	SII
Number of HCC	0.100, p=0.001**	-0.035, p=0.231 ns	0.040, p=0.171	0.112, p<0.001**	0.109, p<0.001**
Size of HCC	0.334, p<0.001**	-0.112, p<0.001**	0.452, p<0.001**	0.340, p<0.001**	0.498, p<0.001**
EHD	0.245, p<0.001**	-0.056, p=0.055 ns	0.230, p<0.001**	0.221, p<0.001**	0.291, p<0.001**
Vascular invasion	0.224, p<0.001**	-0.131, p<0.001**	0.144, p<0.001**	0.267, p<0.001**	0.258, p<0.001**
Constitutional symptoms	0.261, p<0.001**	-0.151, p<0.001**	0.237, p<0.001**	0.305, p<0.001**	0.349, p<0.001**
Cirrhosis	-0.244, p<0.001**	-0.093, p<0.001**	-0.426, p<0.001**	-0.109, p<0.001**	-0.320, p<0.001**
AFP	0.231, p<0.001**	-0.136, p<0.001**	0.202, p<0.001**	0.276, p<0.001**	0.311, p<0.001**
Albumin	-0.068, p=0.020*	0.215, p<0.001**	0.127, p<0.001**	-0.193, p<0.001**	-0.070, p=0.017*
Bilirubin	-0.028, p=0.343 ns	-0.285, p<0.001**	-0.310, p<0.001**	0.187, p<0.001**	-0.053, p=0.069 ns

Table 4.14 Combined cohort – associations of independent variables significantly affecting survival. HCC=hepatocellular cancer; EHD=extra-hepatic disease; AFP=alphafetoprotein; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

Variable	Neutrophils	Lymphocytes	Platelets	NLR	SII
Number of HCC	0.100, p=0.001*	-0.035, p=0.231 ns	0.040, p=0.171	0.112, p<0.001*	0.109, p<0.001*
Size of HCC	0.334, p<0.001*	-0.112, p<0.001*	0.452, p<0.001*	0.340, p<0.001*	0.498, p<0.001*
EHD	0.245, p<0.001*	-0.056, p=0.055 ns	0.230, p<0.001*	0.221, p<0.001*	0.291, p<0.001*
Vascular invasion	0.224, p<0.001*	-0.131, p<0.001*	0.144, p<0.001*	0.267, p<0.001*	0.258, p<0.001*
Constitutional symptoms	0.261, p<0.001*	-0.151, p<0.001*	0.237, p<0.001*	0.305, p<0.001*	0.349, p<0.001*
Cirrhosis	-0.244, p<0.001*	-0.093, p<0.001*	-0.426, p<0.001*	-0.109, p<0.001*	-0.320, p<0.001*
AFP	0.231, p<0.001*	-0.136, p<0.001*	0.202, p<0.001*	0.276, p<0.001*	0.311, p<0.001*
Albumin	-0.068, p=0.020	0.215, p<0.001*	0.127, p<0.001*	-0.193, p<0.001*	-0.070, p=0.017
Bilirubin	-0.028, p=0.343 ns	-0.285, p<0.001*	-0.310, p<0.001*	0.187, p<0.001*	-0.053, p=0.069 ns

Table 4.15 Combined cohort – associations of independent variables significantly affecting survival with Bonferroni correction. HCC=hepatocellular cancer; EHD=extra-hepatic disease; AFP=alphafetoprotein; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

4.6.5 Combined cohort - survival analysis

Univariate and multivariate analyses were carried out as for the previous cohorts **Table 4.16A-C**. In the combined cohort, prognostic factors that remained very highly significant in the multivariate analyses included: HCC tumour size, EHD, PVT, AFP, albumin, INR and treatment ($p<0.001$) **Table 4.16B**. The number of tumours ($p=0.009$), bilirubin ($p=0.002$) and ascites ($p=0.026$) also remained significant too. Of the circulating cell counts and scores, neutrophils and the SII were highly significant independent predictors of prognosis ($p<0.001$) **Table 4.16B-C**. WCC ($p=0.007$) and the NLR ($p=0.005$) were also independent predictor of prognosis **Table 4.16C**.

A. Univariate analysis Variable	Significance
Cirrhosis	0.023
Number of HCC	<0.001
Size of largest HCC	<0.001
EHD	<0.001
PVT	<0.001
Ascites	<0.001
Encephalopathy	0.371
AFP	<0.001
Albumin	<0.001
Bilirubin	<0.001
INR	<0.001
Sodium	0.528
Creatinine	0.822
Rx	<0.001
WCC	<0.001
Neutrophils (cont.)	<0.001
Lymphocytes (cont.)	0.193
Platelets	<0.001
NLR	<0.001
SII	<0.001

B. Multivariate analysis Variable	Significance	HR	95% Confidence Interval	
			Lower	Upper
Number of HCC	0.009	1.042	1.010	1.074
Size of largest HCC	<0.001	1.047	1.031	1.064
EHD	<0.001	1.473	1.232	1.761
PVT	<0.001	1.989	1.693	2.336
Ascites	0.026	1.213	1.023	1.438
AFP	<0.001	1.000	1.000	1.000
Albumin	<0.001	0.963	0.950	0.977
Bilirubin	0.002	1.002	1.001	1.004
INR	<0.001	1.671	1.280	2.180
Rx (OLTx)	<0.001			
Rx (resection)	<0.001	2.968	1.715	5.136
Rx (RFA)	<0.001	5.314	3.283	8.603
Rx (HAT)	<0.001	7.954	5.049	12.530
Rx (Medical)	<0.001	10.476	6.363	17.247
Rx (BSC)	<0.001	15.310	9.692	24.187
WCC	0.006	1.019	1.005	1.033
Platelets	0.487	1.000	1.000	1.001
Neutrophils	<0.001	1.082	1.054	1.111

C. Multivariate analyses - assessing the association of either NLR or SII (other parameters not shown – data very similar to above, in both analyses)				
NLR	0.005	1.015	1.004	1.025
SII	<0.001	1.000	1.000	1.000

Table 4.16 Combined cohort - Cox regression survival analyses. A. Univariate analysis. B-C. Multivariate analysis. Variables that were significant (p<0.01) in a univariate analysis were entered into a multivariate analysis to determine independent variables affecting survival. Combined cohort – differences in OS based on circulating cell count. HCC=hepatocellular cancer; EHD=extra-hepatic disease; PVT=portal vein thrombus; AFP=alphafetoprotein; INR=international normalised ratio; WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index; Rx = treatment; OLTx=orthotopic liver transplant; RFA=radiofrequency ablation; HAT=hepatic arterial therapy; BSC=best supportive care.

From the multivariate analysis, WCC, neutrophils, the NLR and SII were strong independent predictors of prognosis **Table 4.16B-C**. Since lymphocytes and platelets were not significant in the multivariate analysis and the SII – which places more emphasis on neutrophils – was highly statistically significant ($p<0.001$) it can be inferred that neutrophils are the determining factor in terms of circulating inflammatory cells that predict prognosis. Circulating inflammatory cell counts were categorically divided into above and below median or stratified into three categories as previously described.

In the combined cohort, patients with neutrophil counts above median had a shorter median OS (4.4 months, 95% CI 3.7-5.1) compared to patients with a below median neutrophil count (13.6 months, 95% CI 11.7-15.6) Log-Rank $p<0.001$ **Table 4.17** and **Figure 4.15**. The SII could further stratify patients into three groups with those with a high SII (>2000) having the shortest median OS (1.9 months, 95%CI 1.3-2.5); compared to those with a medium SII (SII>500 but <2000) where median OS was 5.7 months (95%CI 4.5-6.8); compared to those with a low SII (<500) where median OS was 15.5 months (95%CI 12.7-18.2), Log Rank, $p<0.001$ **Table 4.17** and **Figure 4.15**.

When circulating cell counts were considered as categorical variables (above or below median, or for the SII divided into three groups), WCC (HR 1.323, 95% CI 1.147-1.527, $p<0.001$); neutrophils (HR 1.390, 95% CI 1.199-1.612, $p<0.001$); lymphocytes (HR 0.743, 95% CI 0.643-0.852, $p=0.002$); NLR (HR 1.478, 95% CI 1.283-1.703, $p<0.001$) and SII (medium SII HR 1.244, 95% CI 1.070-1.447, $p=0.005$; high SII HR 2.185, 95% CI 1.721-2.774, $p<0.001$) remained independent variables affecting survival in multivariate analyses **Table 4.18**.

Circulating immune cell/score	Months < median	95% CI		Months > median	95% CI		p-value
		Lower	Upper		Lower	Upper	
WCC	11.5	9.6	13.4	5.3	4.2	6.4	<0.001
Neutrophils	13.6	11.7	15.6	4.4	3.7	5.1	<0.001
Lymphocytes	5.6	4.5	6.7	11.5	9.5	13.5	<0.001
Platelets	11.9	10.1	13.7	5.3	4.4	6.3	<0.001
NLR	15.1	12.9	17.3	4.0	3.4	4.5	<0.001
SII	Months survival			p-value			
SII<500	15.5	12.7	18.2	<0.001			
SII>500<2000	5.7	4.5	6.8				
SII>2000	1.9	1.3	2.5				

Table 4.17 Combined cohort – Kaplan-Meier survival analysis. Median survival in months in patients with above or below median circulating inflammatory cell counts or scores. Median WCC = 6.6; median neutrophil count = 4.2; median lymphocyte count = 1.3; median platelet count = 172; median NLR = 3.2; SII was divided into three categories: SII<500, SII>500 but <2000, SII>2000). Upper and lower 95% confidence intervals are shown along with Log-Rank p-values.

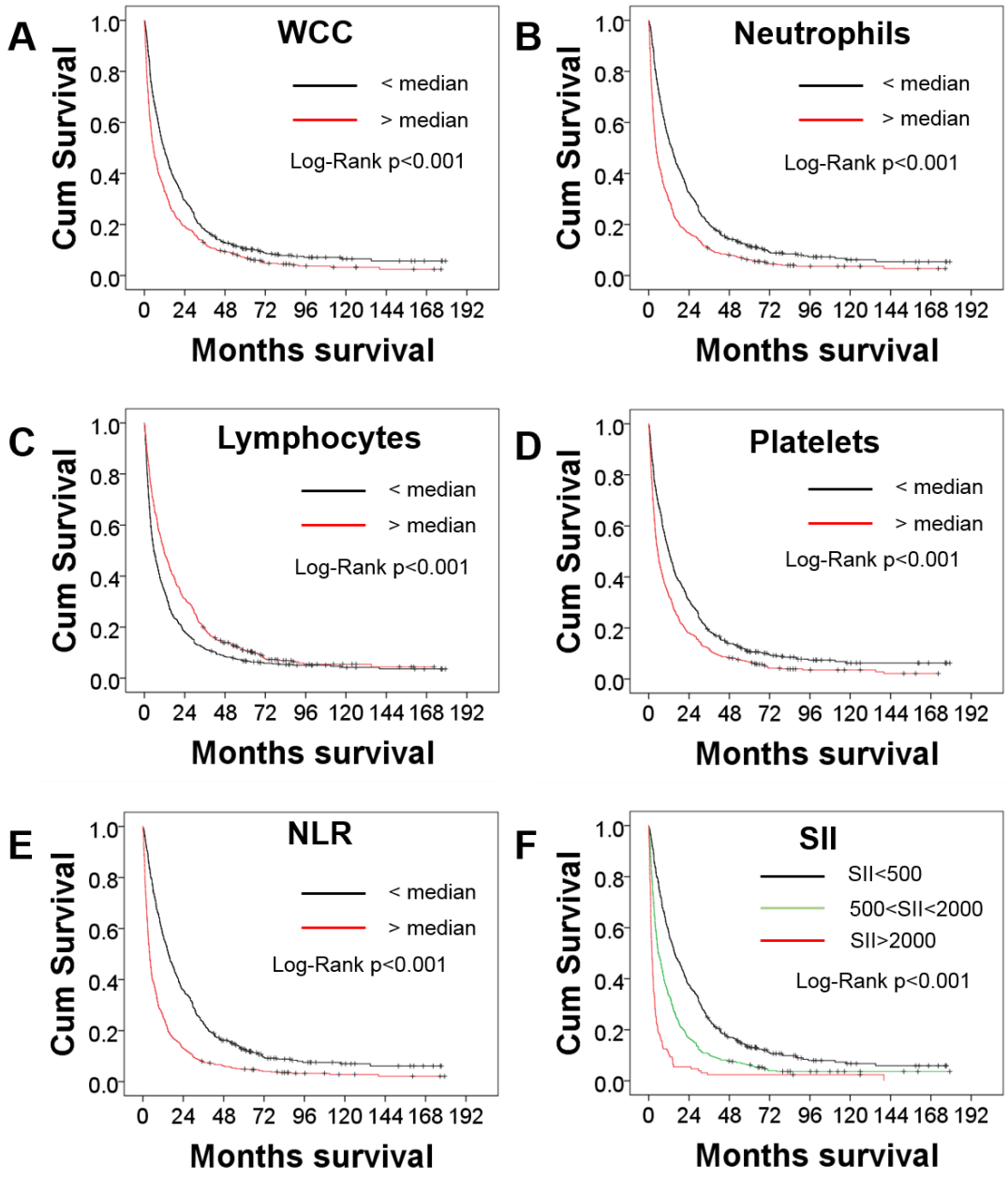


Figure 4.15 Combined cohort - Kaplan-Meier survival curves to show to differences between patients with above or below median A. neutrophil count B. lymphocyte count C. platelet count D. neutrophil to lymphocyte ratio. E. Patients were divided into three groups on the basis of having a low (<500), medium (SII>500 but <2000) or high (>2000) SII. NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

A. Univariate analysis Variable	p-value
WCC (cat.)	<0.001
Neutrophils (cat.)	<0.001
Lymphocytes (cat.)	<0.001
Platelets (cat.)	<0.001
NLR (cat.)	<0.001
SII (cat.)	<0.001

B. Multivariate analysis Variable	p-value	HR	95% Confidence Interval	
			Lower	Upper
WCC (cat.)	<0.001	1.323	1.147	1.527
Neutrophils (cat.)	<0.001	1.390	1.199	1.612
Lymphocytes (cat.)	<0.001	0.743	0.648	0.852
Platelets (cat.)	0.661	0.965	0.825	1.130
NLR (cat.)	<0.001	1.478	1.283	1.703
SII <500 (cat.)	<0.001			
SII>500 but <2000 (cat.)	0.005	1.244	1.070	1.447
SII>2000 (cat.)	<0.001	2.185	1.721	2.774

Table 4.18 Combined cohort – Cox regression analysis A. Univariate. B. Multivariate. Cell counts as categorical variables were also analysed. Data were categorised into above or below median values (median WCC = 6.6; median neutrophil count = 4.2; median lymphocyte count = 1.3; median platelet count = 172; median NLR = 3.2; SII was divided into three categories: SII<500, SII>500 but <2000, SII>2000). Neutrophils, lymphocytes and platelets were significant <0.01 in univariate analyses and were entered into a multivariate analysis containing the variables that were significant in the univariate analyses in Table 4.16.

4.6.6 Exploration of circulating inflammatory cell counts and scores in individual patient groups

The combined cohort was used to investigate the use of circulating inflammatory cell counts/ scores in individual patient groups – divided on the basis of primary treatment. The purpose of this was to explore the clinical utility of such cell counts/scores in predicting prognosis in terms of median OS. Since neutrophils, the NLR and SII remained statistically significant in the combined cohort in a multivariate Cox analysis, these were tested across different treatment groups and groups of patients divided on the basis of BCLC stage.

4.6.7 The use of circulating neutrophil count, NLR and SII across different treatment groups

The clinical utility of neutrophils, the NLR and SII were explored in the following treatment groups: orthotopic liver transplant, resection, ablation, arterial (embolization or SIRT), systemic medical therapy and in patients where best supportive care was provided. In a Kaplan-Meier survival analysis, circulating neutrophil counts, the NLR and SII were effective at showing statistically different median OS times in months in patients receiving arterial and best supportive care **Table 4.19-Table 4.21**. The SII also produced the most significant (Log-Rank p=0.011) prognosticator in terms of systemic/medical therapy **Table 4.21** and the NLR also differentiated patients' median OS receiving systemic/medical therapy (Log-Rank p=0.020) **Table 4.19.** Neutrophils, the NLR and SII all failed to demonstrate difference in median OS for patients receiving surgical treatment.

Treatment	Months survival Neutrophils <median	95% Confidence Interval		Months survival Neutrophils> median	95% Confidence Interval		p-value
		Lower	Upper		Lower	Upper	
OLTx	117.5	-	-	87.8	-	-	0.838
Resection	31.5	25.6	37.4	61.4	44.8	78.0	0.054
Ablation	30.2	27.1	33.3	30.8	18.7	42.9	0.561
Arterial treatment	17.2	14.0	20.4	17.3	9.4	12.5	<0.001
Systemic/medical	6.2	2.3	10.0	4.5	3.6	5.4	0.044
Supportive care	4.9	3.8	6.0	1.8	1.4	2.2	<0.001

Table 4.19 Kaplan-Meier survival analysis. Differences in median OS (months) in patients with above or below median circulating neutrophil counts were assessed in different treatment groups. OLTx=orthotopic liver transplant.

Treatment	Months survival NLR <median	95% Confidence Interval		Months survival NLR >median	95% Confidence Interval		p-value
		Lower	Upper		Lower	Upper	
OLTx	-	-	-	79.9	26.1	133.8	0.337
Resection	37.9	24.6	51.2	52.4	11.5	93.2	0.238
Ablation	31.4	28.6	34.2	25.4	8.6	42.2	0.915
Arterial treatment	17.6	14.6	20.6	9.2	6.8	11.6	<0.001
Systemic/medical	6.6	1.9	11.2	4.6	3.7	5.5	0.020
Supportive care	5.8	4.3	7.3	2.1	1.8	7.3	<0.001

Table 4.20 Kaplan-Meier survival analysis. Differences in median or *mean OS (months) in patients with above or below median NLR counts were assessed in different treatment groups. OLTx=orthotopic liver transplant.

Treatment	Months survival SII<500	95% Confidence Interval		Months survival SII>500 but <2000	95% Confidence Interval		Months survival SII >2000	95% Confidence Interval		p-value
		Lower	Upper		Lower	Upper		Lower	Upper	
OLTx	-	-	-	-	-	-	-	-	-	-
Resection	31.5	24.6	38.4	61.4	43.7	79.1	35.0	-	-	0.025
Ablation	31.0	28.7	33.3	26.7	10.7	42.6	12.0	-	-	0.386
Arterial treatment	19.1	15.6	22.7	11.4	9.1	13.7	4.5	2.2	6.8	<0.001
Systemic/medical	6.0	3.8	8.2	5.1	4.4	5.9	3.7	1.6	0.5	0.011
Supportive care	5.8	4.1	7.5	2.5	2.0	2.9	1.3	1.1	1.5	<0.001

Table 4.21 Kaplan-Meier survival analysis. Differences in median OS (months) in patients with low (<500), medium (SII>500 but <2000) or high (>2000) were assessed in different treatment groups. SII=systemic immune-inflammation index; OLTx=orthotopic liver transplant.

4.6.8 BCLC groups

The prognostic utility of neutrophils, NLR and SII was also explored in patients divided on the basis of their BCLC stage (0/A; B; C or D). Circulating neutrophil count, the NLR and SII were able to differentiate BCLC grade C/D patients' prognostically in terms of median OS with statistical significance **Table 4.22-Table 4.24**. The NLR was the only score where this extended to patients staged as BCLC B (Log-Rank p=0.007) **Table 4.23**. None of the scores were able to prognosticate patients that were BCLC 0/1 in terms of median OS.

BCLC stage	Months survival Neutrophils< median	95% Confidence Interval		Months survival Neutrophils >median	95% Confidence Interval		p-value
		Lower	Upper		Lower	Upper	
BCLC 0/A	42.7	36.0	49.4	36.2	7.0	65.3	0.949
BCLC B	20.6	16.7	24.6	17.8	12.3	23.3	0.372
BCLC C	8.2	6.8	9.6	3.8	3.2	4.3	<0.001
BCLC D	4.9	3.5	6.2	1.5	1.1	2.0	0.004

Table 4.22 Kaplan-Meier survival analysis. Differences in median OS (months) in patients with above or below median neutrophils were assessed in patients with different BCLC stages. BCLC=Barcelona Clinic for Liver cancer.

BCLC stage	Months survival NLR< median	95% Confidence Interval		Months survival NLR> median	95% Confidence Interval		p-value
		Lower	Upper		Lower	Upper	
BCLC 0/A	41.8	35.6	48.0	52.4	35.6	69.1	0.759
BCLC B	22.6	17.7	27.4	14.9	11.1	18.7	0.007
BCLC C	9.5	7.9	11.1	3.4	2.8	4.0	<0.001
BCLC D	5.8	4.3	7.3	1.6	1.0	2.2	<0.001

Table 4.23 Kaplan-Meier survival analysis. Differences in median OS (months) in patients with above or below median NLR were assessed in patients with different BCLC stages. BCLC=Barcelona Clinic for Liver cancer.

BCLC stage	Months survival SII<500	95% Confidence Interval		Months survival SII>500 but <2000	95% Confidence Interval		Months survival SII >2000	95% Confidence Interval		p-value
		Lower	Upper		Lower	Upper		Lower	Upper	
BCLC 0/A	42.3	34.7	49.8	52.4	16.5	88.3	42.5	35.0	50.0	0.747
BCLC B	22.5	18.9	26.1	16.8	13.9	19.6	6.2	0.0	15.6	0.301
BCLC C	8.5	6.7	10.4	5.2	4.0	6.4	2.0	1.4	2.5	<0.001
BCLC D	6.1	4.5	7.7	1.7	1.1	2.4	1.2	1.1	1.3	<0.001

Table 4.24 Kaplan-Meier survival analysis. Differences in median OS (months) in patients with low (<500), medium (SII>500 but <2000) or high (>2000) were assessed in patients with different BCLC stages. BCLC=Barcelona Clinic for Liver cancer.

4.6.9 Clinical Utility of Inflammatory Scores within the BCLC Stage

While the associations with individual cell types possibly allude to biological mechanisms associated with disease progression, the data described above supports additional discriminatory value in terms of clinical utility. The BCLC staging system is the one most commonly employed to aid management decisions and choice of therapy, although it does have associated limitations. The data in **Table 4.24** suggest that in patients with BCLC-C or BCLC-D disease, the neutrophil count, the NLR and the SII are highly significantly associated with outcome. This information is not likely to be helpful for patients with BCLC-D disease (n=168), as there is no therapy available other than supportive care. We have explored further, however, in patients classed as BCLC-C - making up the largest group in this combined cohort (n=584). This stage is also the most heterogeneous, as patients may be classed as BCLC-C based on advanced tumour stage with preserved liver function, early or intermediate stage tumour but with deteriorating liver function, or with early/intermediate disease but with deteriorating ECOG performance status. Each of these factors (tumour, liver function, PST) are clearly important in terms of survival, and at the time the BCLC stage was created, there were no evidence based treatment options reportedly improving survival in this category and thus these patients were recommended for supportive care or clinical trials. This was not always acceptable in practice and many centres strove to offer transplant for those with early cancers, while others offered loco-regional therapy where liver function was relatively preserved, in the presence of a partial PVT, or even with a suspicion of small volume EHD. The subsequent introduction of medical therapy with sorafenib did change the options for these cases, and the BCLC algorithm has been revised recommending sorafenib first line for BCLC-C patients. Many of the patients in this study however predated the introduction of sorafenib, and the breakdown of treatments were as shown in **Table 4.25**.

Treatment BCLC-C	Number	Survival (median months)	Whole cohort number	Whole cohort Median survival
Transplant	16 (16)	82.0	53 (53)	112.4
Resection	13 (8)	32.0	99 (51)	41.3
Ablation	28 (27)	18.0	104 (89)	30.3
Arterial	155 (151)	10.2	317 (296)	14.5
Medical	110 (108)	7.0	137 (131)	7.6
Supportive	262 (251)	7.2	458 (441)	7.2
Overall	584 (561)	8.3	1168 (1061)	10.2

Table 4.25 Primary treatment of BCLC-C patients (n=584). The number of patients receiving each treatment regimen are given with the numbers in brackets representing those patients that had complete survival analysis data and were included in survival analyses.

While the median survival of the BCLC-C stage was just 8.3 months, there was clearly a very wide range of treatments administered, and a wide range in terms of survival (median 7.2 – 82.0 months). There is no doubt that within the BCLC-C stage, many patients benefited from treatment. Survival within the different treatment categories for the whole cohort is also shown for comparison and while there was no difference in those receiving medical therapy or supportive care, there were differences in those receiving surgical or loco-regional treatments. The challenge remains selecting those that would benefit and avoiding treating those that would not. In this cohort, the numbers treated surgically were relatively small, but we have focused further on the BCLC-C cases receiving loco-regional therapy. Data analysed by univariate Cox Regression analysis are shown in **Table 4.26A**.

A. Univariate analysis Variable (BCLC-C receiving arterial therapy)	Significance
Cirrhosis (98/53)	0.949
Number of HCC	<0.001
Size of largest HCC	0.247
EHD (132/19)	0.691
PVT (118/33)	0.017
Ascites (120/31)	0.573
Encephalopathy (107/2)	0.516
AFP	0.014
Albumin	0.639
Bilirubin	0.289
INR	0.493
Sodium	0.002
Creatinine	0.393
ECOG PST	0.300
WCC (cont.)	0.407
Neutrophils (cont.)	0.005
Platelets (cont.)	0.075
Lymphocytes (cont.)	0.096
NLR (cont.)	0.003
SII (cont.)	0.001
Neutrophils (cat.)	0.003
Platelets (cat.)	0.194
Lymphocytes (cat.)	0.069
NLR (cat.)	<0.001
SII (cat.)	0.013

B. Multivariate analysis Variable	Significance	HR	95% Confidence Interval	
			Lower	Upper
Number of HCC	0.001	1.135	1.051	1.225
Sodium (cont)	0.001	0.902	0.849	0.958
Neutrophils (cont.)	0.744	0.984	0.894	1.084
NLR (cont.)	0.671	1.017	0.940	1.101
SII (cont.)	0.671	1.000	1.000	1.000

C. Multivariate analyses – neutrophils, NLR & SII (categorical)				
Number of HCC	<0.001	1.120	1.057	1.187
Sodium	0.004	0.911	0.855	0.971
Neutrophils (cat.)	0.423	1.196	0.772	1.854
NLR (cat.)	0.318	1.243	0.811	1.906

Table 4.26 Cox regression analysis in BCLC-C patients. A. Univariate analysis of clinical parameters. B. Multivariate analysis including AFP and SII as continuous variables. C. Multivariate analysis including AFP as a continuous variable and SII as categorical variable. HCC=hepatocellular cancer; EHD=extra-hepatic disease; PVT=portal vein thrombus; AFP=alphafetoprotein; INR=international normalised ratio; ECOG PST= Eastern cooperative oncology group performance status; WCC=white cell count; NLR=neutrophil to lymphocyte ratio; SII=systemic immune-inflammation index.

As cases were classed as BCLC-C, they were more homogenous for liver function, tumour stage and PST, with far fewer factors showing a significant associations with

survival in the univariate analysis. Restricting to only those variables with a p-value <0.01, the number of tumours, the level of sodium, neutrophils, the NLR and the SII were analysed by multivariate analysis **Table 4.26B-C**. Only tumour number and sodium remained significant multivariate analyses in this sub-cohort of patients.

4.7 Discussion

The Newcastle cohort was the first cohort to be investigated for the relevance of circulating inflammatory cell counts and scores. In this cohort, it was found that there were trends between TNM stage, Child-Pugh, BCLC and ECOG performance status with circulating inflammatory cell counts and scores. Circulating inflammatory cells counts generally increased with more advanced stage or grade except in the case of lymphocytes, where lymphocytes decreased with increasing Child-Pugh grade, advanced BCLC stage and worse ECOG PST. Platelets also decreased with advanced Child-Pugh stage. Similar patterns were observed in the Hong Kong cohort.

In terms of associations with key HCC prognostic factors (number of nodules, HCC size, EHD, cirrhosis, vascular invasion, constitutional symptoms, AFP, albumin and bilirubin), there were similar patterns observed in the two cohorts, the majority of which reached high statistical significance in the combined cohort. When assessing the prognostic implications of circulating cell counts and scores in a Cox multivariate analysis, peripheral blood neutrophil count was the only cell type to remain significant in the individual cohorts (Newcastle $p=0.015$; Hong Kong $p<0.001$; combined $p<0.001$). The NLR and SII as categorical variables ($p<0.001$) were also significant in multivariate analyses in the Newcastle and combined cohort. As categorical variables, in the Newcastle cohort, neutrophils, the NLR and SII remained independent in multivariate analyses and demonstrated statistically significant differences in median OS. Similarities were seen in the Hong Kong and combined cohorts. In the former (Hong Kong) above or below median lymphocyte counts remained significant in a multivariate analysis ($p=0.017$) indicating that perhaps lymphocytes have a greater role in viral hepatitis induced HCC. In the latter, all cell counts/scores were highly significant as categorical variables except platelets.

In the combined cohort which consisted of patients with a more varied aetiology, disease stage and treatment regimen; circulating neutrophil counts, the NLR and SII remained statistically significant in a multivariate analysis. Lymphocytes were significantly

associated with survival in a univariate analysis but did not remain so in the multivariate analysis. Furthermore, the NLR, although significant (p=0.002) was not as highly significant as circulating neutrophil counts and SII (p<0.001). Since the SII score places more emphasis on neutrophils in the calculation of this score, together with these findings it is suggested that it may in fact be neutrophils that are the key drivers of HCC progression in terms of inflammatory cells as previously concluded in literature (Piccard et al., 2012, Sionov et al., 2015, Xu et al., 2014).

Exploration in the combined cohort within individual treatment groups demonstrated that circulating neutrophil count, NLR and SII were associated with statistically significant differences in median OS in patient groups where the primary treatment was loco-regional, systemic/sorafenib or where no treatment was instigated (best supportive care). When patients were divided on the basis of BCLC stage, there were similar significant prognostic associations in patients with BCLC-C or BCLC-D disease, although none of the counts or scores showed any additional discriminatory benefit in in patients that were staged as BCLC 0/A. Focusing on those cases classed as BCLC-C who were treated with loco-regional therapy, our study suggests that the number of tumours, the AFP and a raised SII could be used as additional factors to stratify treatment – although optimal cut-offs have yet to be determined.

In the literature Xu and colleagues demonstrated a correlation with CTCs and SII (Xu et al., 2014). A similar relationship was not observed when exploring this in the Newcastle HCC CTC cohort used in the previous **Chapter 3** (n=69); possibly due to the low sample size. Interestingly, there were indications of an association between epithelial biomarker negative CTC and numbers of neutrophils (0.240, p=0.049) in the peripheral blood count taken on the same day. In addition, numbers of biomarker negative CTCs were weakly associated with the presence of PVT (0.348, p=0.004; Spearman's Rho test). 12/14 patients with a PVT had CTC detected and in 10 of these cases, CTCs were epithelial biomarker negative.

In summary, these cohort studies support a key role for neutrophils – either as a driver or at least as the most significant single peripheral blood cell count – associated with more advanced disease and poorer survival for patients with HCC. As such, the potential role of neutrophils as a driver of tumour progression or possible suppressor of the anti-tumour responses of other immune cells, would seem worthy of pursuit. In terms of clinical utility, the combination scores generally had greater hazards ratios and were considered more likely to have a clinical use. In particular, the SII may have value in the stratification of loco-regional versus medical therapy in patients with BCLC-C stage HCC. Categorical grouping of patients into above or below SII 500 demonstrated significant differences in OS (SII>500=9.9 months versus SII<500=12.5 months, HR1.6, Log-Rank p=0.002). Returning to potentially relevant biology, in the next chapter (**Chapter 5**), gene expression analysis will be carried out on PBMCs in attempt to elucidate altered gene expression that may contribute to hepatocarcinogenesis.

4.8 Conclusions

- Increasing neutrophils, NLR and SII are associated with a more advanced tumour stage, declining liver function and a worse performance status. Conversely increasing lymphocyte counts were associated with less advanced stage of HCC, less extensive underlying liver disease and performance status.
- Counts of circulating neutrophils and the SII were independent prognostic indicators of survival in all cohorts indicating that irrespective of aetiology, neutrophils may be key players in the inflammatory response and associated tumourigenicity in HCC.
- Investigating the prognostic value of circulating neutrophils, the NLR and SII demonstrated that the NLR was able to determine differences in median OS in patients with BCLC B-D stage HCC and categorically grouping patients based on their SII followed by circulating neutrophil count was able to differentiate patients with shorter or longer median OS.

Chapter 5. Peripheral blood mononucleocyte gene expression in NAFLD/NASH-HCC patients

5.1 Introduction

5.1.1 Biomarkers in NAFLD/NASH-HCC

One of the major problems with identifying universal biomarkers in HCC is that the aetiology of disease is highly variable geographically. In Africa and Asia the majority of cases of HCC develop on a HBV aetiology whereas in the US and Europe, leading risk factors include HCV and NASH (Spengler and Loomba, 2015). In Newcastle, ALD and NAFLD are the two most common aetiologies of CLD associated with HCC development (Dyson et al., 2014). It is expected that cases of CLD caused by viral infections will decrease due to the implementation of HBV immunisation and the introduction of interferon therapy for HCV (Mahoney, 1999, Camma et al., 2001). On the other hand, metabolic risk factors – including obesity and type 2 diabetes – associated with NAFLD/NASH-HCC are rising and it is predicted that this trend is only going to increase in the future (Dyson et al., 2014). Variations in HCC aetiology indicate that there will be differences in gene alterations within different patient cohorts since the pathogenesis of disease is variable. Currently, despite the recommendation for surveillance programmes in those with cirrhosis who are at higher risk, many patients present either symptomatically or incidentally (Dongiovanni et al., 2014, Dyson et al., 2014). There are a number of reasons for this. Six monthly abdominal ultrasound (USS), with or without serum AFP measurement, is the most commonly implemented methodology, but actually has poor sensitivity for early disease detection. Despite 6 monthly abdominal USS being promoted by international bodies such as EASL and AASLD (Bruix and Sherman, 2011, Bruix et al., 2001), direct evidence for its cost effectiveness is lacking and even in affluent societies it is poorly implemented. There is widespread recognition that better surveillance tests are needed.

In fact surveillance fails NAFLD patients even more than it does those with other aetiologies of CLD (Dyson et al., 2014). A successful programme relies on the identification of the at risk population. In NAFLD, cirrhosis is often undetected prior to the development of HCC and the opportunity to perform surveillance is missed. In addition, USS is less sensitive in patients with obesity, so the test is even less sensitive. And in fact, many patients with NAFLD develop HCC on a background of obesity, type 2 diabetes and fatty liver without significant fibrosis. For these individuals, without cirrhosis, there is presently no means of stratifying HCC risk and no recommendation at all for HCC surveillance given the size of the affected population and the lack of effective tools. There is, however, a drive to identify suitable surveillance biomarkers for this HCC aetiology. While the use of non-invasive tests to monitor disease progression in NAFLD/NASH patients is appealing, aiming to detect early disease and increase the possibility of patients receiving curative treatment (Ascha et al., 2010, Dongiovanni et al., 2014, Michelotti et al., 2013), there are limited reports of novel circulating biomarkers for use in the clinical management of NAFLD/NASH HCC. Several studies have investigated the use of measuring markers of liver function including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and the AST:ALT ratio in combination with metabolic factors to differentiate NASH from other chronic liver disease (Gholam et al., 2007, Sorbi et al., 1999). Data has also demonstrated that high plasma levels of CK-18 is independently associated with the presence of NASH (Feldstein et al., 2009).

5.1.2 PBMC genomic profiling

Recently, there has been an increase in the development of 'liquid biopsies'. Blood is an accessible and clinically routine sampling method. Blood draws are minimally invasive and provide relatively low discomfort to patients compared to tissue biopsies. In addition to the identification, quantification and characterisation of CTCs; it has been considered that other components of the blood may be able to provide clinically useful information: including PBMCs consist of lymphoid cells (T-cells, B-cells, NK cells) and myeloid cells (monocytes, granulocytes, neutrophils, eosinophils, basophils and dendritic

cells). Due to the inflammatory nature of HCC and immune involvement, it is hypothesised that there will be phenotypic differences in circulating immune cells from HCC patients with a NAFLD/NASH aetiology compared to respective matched controls without HCC, and that these might be exploited as early diagnostic or surveillance signatures.

5.1.3 Development of PBMC genes signatures in HCC

In a previous pilot study carried out by the Hui group in Singapore, a 3-gene blood signature was developed that could identify early HCC (BCLC 0 or A) in patients that did or did not have elevated serum AFP (≥20 ng/ml) and could distinguish between patients with HCC from healthy volunteer samples and patients with chronic hepatitis B (Shi et al., 2014). The approach taken to identify this gene signature included microarray analysis of RNA extracted from PBMCs from 10 HCC patients, 10 healthy volunteers, 3 pancreatic cancer patients and 3 gastric cancer patients (Shi et al., 2014). From this initial analysis, 6 genes were found to be differentially expressed in PBMCs from HCC patients (Shi et al., 2014). Next, validation of these 6 genes was attempted using multiplex PCR and univariate analysis, resulting in RAB18 – a member of the RAS oncogene family – dropping out due to lack of significance in a univariate analysis (Shi et al., 2014). Of the 5 remaining genes, 3 were able to distinguish between HCC and non-HCC cases (CXCR2 – chemokine C-X-C motif receptor 2 – an IL-8 receptor; CCR2 – chemokine receptor 2 which is a receptor for monocyte chemoattractant protein-1; and EP400 - E1A-Binding Protein P400, a chromatin re-modelling protein) (Shi et al., 2014, NCBI Gene Database, 2016).

In **Chapter 4**, counts and ratios of immune cells in HCC patients were explored, concluding that neutrophils have a key role in HCC tumourigenesis and that the SII has independent prognostic utility. In this chapter, differentially expressed genes in PBMCs were explored in HCC patients with NAFLD/NASH aetiology and compared to that of NAFLD/NASH cirrhotic controls as well as a HBV-HCC set of patients. Pilot data presented in this chapter is the beginning of a collaborative study with the Hui research

group in Singapore. The long term view is that continued sample collection will lead to the generation of a training cohort to assess the possibility of generating a gene signature that could detect early stage disease or have prognostic value in NAFLD/NASH-HCC patients.

5.2 Materials and Methods

5.2.1 Extraction of peripheral blood mononucleocytes (PBMCs) from HCC patient blood samples

8 ml blood samples were collected in EDTA tubes from patients with NAFLD/NASH cirrhosis and patients that had HCC developed on a background of NAFLD/NASH under the care of Dr Quentin Anstee, Newcastle, UK. Samples were obtained following ethical approval obtained by the Newcastle Hepatopancreatobiliary and Gastroenterology Research Tissue Bank. All patients used in testing had histological confirmation of NAFLD/NASH. Samples were transported on ice and processed within 2 h of collection. Samples were subjected to density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences) to form distinguishable layers of RBCs, density medium, PBMCs and plasma. The PBMCs were removed and pelleted after two 10 min centrifugations at 100 x g with 10 ml PBS. The PBMC pellet was re-suspended in 300 µl of TRIzol® (Life Technologies) and stored at -80 ^{o}C prior to shipping.

5.2.2 Gene Microarray

Data presented in this chapter is kindly provided by Professor Kam Hui and colleagues (Dr H. Xia, Mr S.V. Pratap and Mr S. Karthik) as part of a collaborative study. Samples were shipped on dry ice to Professor Kam Hui, National Cancer Centre Singapore where gene microarray and analyses were performed. RNA was extracted to perform microarray analysis to look at the gene expression profile in PBMCs from HCC patients compared to the control group. A further group of HCC patients with a HBV aetiology were included for further comparison. The gene microarray was performed using the Illumina HumanHT-12 v4 Expression BeadChip platform (Illumina, US). The HT-12 v4 BeadChip provides genome wide coverage of 31,000 genes with >47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (November 7, 2009). An overview of the method is given in **Figure**

5.1. 12 blood samples were assessed consisting of 4 NAFLD/NASH control patients, 4 NAFLD/NASH-HCC patients and 4 HBV-HCC patients.

5.2.3 Statistical Analysis

Differentially expressed genes were identified using analysis of variance (ANOVA), (p-value <0.05) between NAFLD controls, NAFLD-HCC samples and HBV-HCC samples. Heat map with hierarchal clustering analysis and principal component analysis (PCA) were carried out using Partek Genomics Suite 6.5 software. Ingenuity® pathway analysis (Qiagen) was performed to identify associations between differentially expressed genes, associated molecules and signalling pathways.

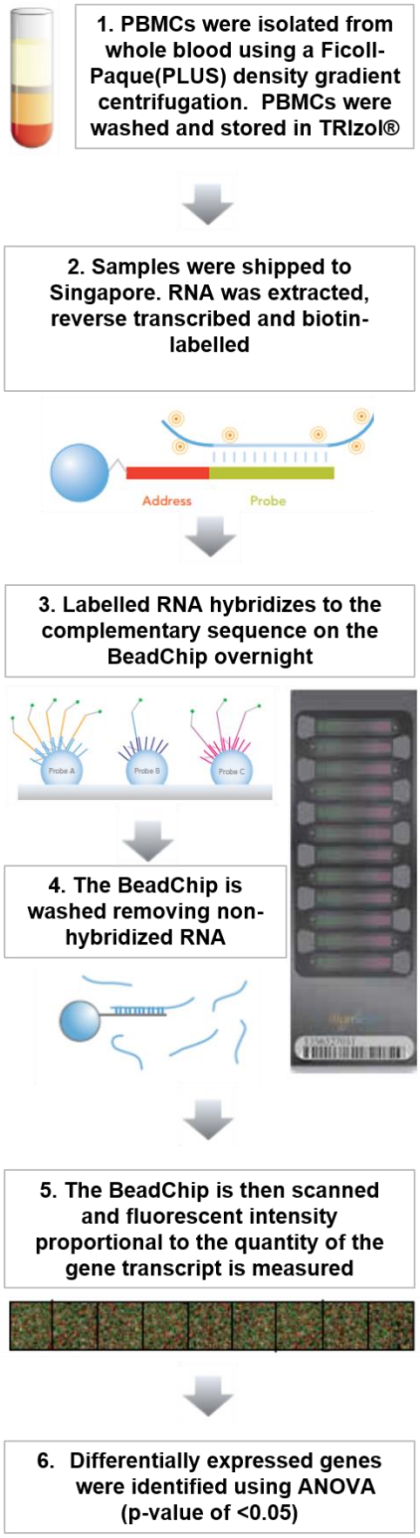


Figure 5.1 Overview of sample processing for the Illumina HumanHT-12 v4 Expression BeadChip platform. Adapted from Illumina 2010.

5.3 Aims

- Identify differentially expressed genes between NAFLD/NASH controls and NAFLD/NASH-HCC patients in a pilot cohort of cirrhotic controls and HCC patients
- Explore the role of up-/down-regulated genes identified by the gene microarray in HCC

5.4 Results

5.4.1 Differential gene expression between NAFLD/NASH control, NAFLD/NASH-HCC and HBV-HCC patient samples

Results of the PBMC microarray using the Illumina HumanHT-12 v4 Expression BeadChip platform which covers 31,000 genes showed that there was differential gene expression between the three sample groups. Differentially expressed genes were defined as those with a fold change of >1.5 and a p-value of <0.05 using ANOVA to assess difference between the sample groups. There were 523 differentially expressed genes between NAFLD/NASH control samples compared to NAFLD/NASH HCC. Between the HBV-HCC PBMC samples and the NAFLD/NASH HCC samples there were 604 differentially expressed genes. Heat map analysis using unsupervised hierarchal clustering demonstrated that there were more similarities between NAFLD/NASH-HCC and HBV-HCC **Figure 5.2**.

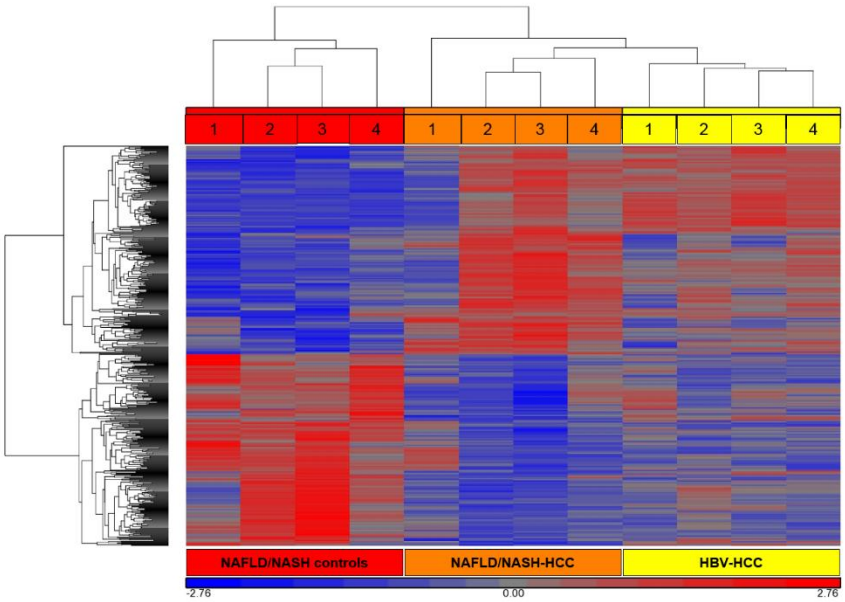


Figure 5.2 Heat map analysis demonstrating differentially expressed genes between NAFLD/NADH cirrhotic controls (red), NAFLD/NASH-HCC patients (orange) and HBV-HCC patients (yellow). Upregulated genes are coded in red whereas down-regulated genes are denoted in blue.

5.4.2 More variation was observed between NAFLD/NASH controls and NAFLD/NASH HCC compared to HBV-HCC and NAFLD/NASH HCC

Principal components analysis (PCA) was used to identify key variations in the data **Figure 5.3**. Variations in the data were expressed as eigenvalues as a percentage of the total variation. The first principal component separated samples on the x-axis accounted for 63.4% of the variance demonstrating that there were larger variations in the data between NAFLD/NASH controls and NAFLD/NASH HCC patients. The second principal component (y-axis) accounted for 16.4% of the total variance and distinguished HBV-HCC from NAFLD/NASH-HCC. The third principal component separated samples out on the z-axis, although this component only accounted for 7.16% of the variance. To conclude, control samples were most variable between the two different HCC aetiologies (HBV and NAFLD/NASH) and this is also demonstrated in the hierarchal clustering analysis on the heat map **Figure 5.2**. However, the HBV-HCC aetiology clustered

distinctly from the NAFLD/NASH-HCC aetiology although there was less variance between these two sample groups.

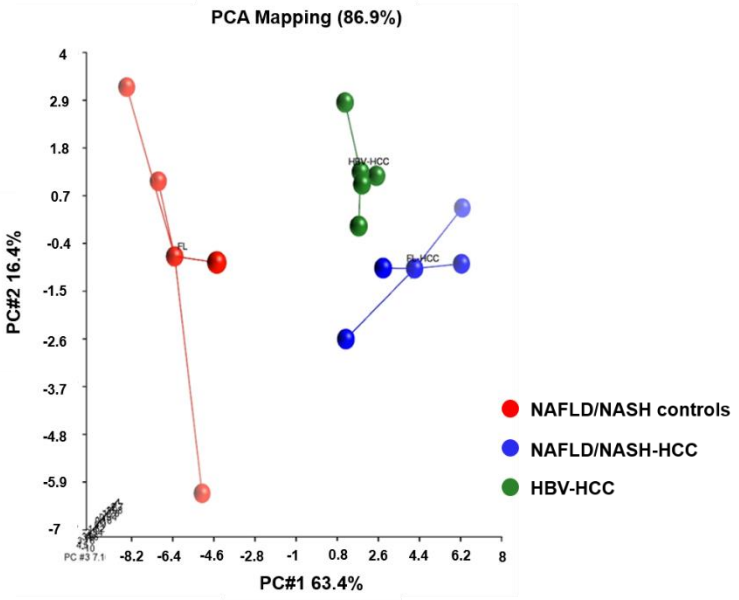


Figure 5.3 PCA analysis demonstrated that NAFLD/NASH HCC was more similar to HBV-HCC than NAFLD/NASH controls in terms of differential gene expression in PBMCs.

5.4.3 Altered gene pathways in NAFLD-NASH-HCC

Ingenuity® is a software program that can be used to explore relationships between differentially expressed genes in order to assess the biological relevance. In the gene microarray data from the PBMCs, the top altered pathways are listed in **Table 5.1**. The role of nuclear factor of activated T-cells (NFAT) associated with the immune response was identified as the top most altered pathway and is discussed further in **Section 5.4.4**. Of the other top altered pathways identified, integrin signalling was identified as the second most altered pathway using Ingenuity®. Integrin association with RTKs leads to the activation of pathways involved in tumour cell invasion and metastasis; aiding in cell detachment, migration and survival and initiation of proliferation at distal sites in a different microenvironment (Guo and Giancotti, 2004). Gene alterations in phospholipase-C signalling were also detected which have been shown to generate lipid

mediators responsible for metastatic processes including cell migration, invasion and angiogenesis in addition to proliferation (Park et al., 2012) . Genes involved in the degradation of 3-phosphoinositide (PI3K) were upregulated **Table 5.1**. Lastly, genes (TAF1C, POLR1C) associated with the assembly of RNA polymerase I complex required for formation of the pre-initiation complex (PIC) which is in turn required for initiation of transcription were downregulated .

Pathway	p-value	Altered genes
Role of NFAT in Regulation of the Immune Response (10 / 160)	0.005	GSK3B, GNAZ, FYN, PPP3R1, ITPR3, GNG11, CD79B, MEF2D, MAPK3, FCGR3A/FCGR3B
Phospholipase C Signalling (11 / 221)	0.017	PPP1R14A, ARHGEF3, FYN, PPP3R1, ITPR3, GNG11, CD79B, RHOT1, MYL9, MEF2D, MAPK3
Integrin Signalling (10 / 194)	0.018	ITGA2B, GSK3B, CAPN3, FYN, CTTN, RHOT1, ITGB3, MYL9, ACTN1, MAPK3
3-phosphoinositide Degradation (8 / 139)	0.018	DUSP10, ALPL, DUSP8, PPP1R14A, PPP2R5A, PTPN22, INPP4A, DUSP16
Assembly of RNA Polymerase I Complex (2 / 9)	0.018	TAF1C, POLR1C

Table 5.1 Top altered pathways in NAFLD/NASH-HCC identified using Ingenuity®. Upregulated genes are denoted in red and downregulated genes are denoted in green.

5.4.4 The role of NFAT in the regulation of the immune response - the top altered candidate pathway in NAFLD/NASH-HCC

In the PBMC gene microarray, the role of NFAT in the regulation of the immune response was the top altered pathway in NAFLD/NASH-HCC. The five members of the NFAT family of transcription factors are essential to T-cell development and function but the role of different NFAT isoforms have been implicated in a range of different cancers including breast, pancreatic and lymphoma (Macian, 2005, Pan et al., 2013). In cancer, evidence of NFAT overexpression, its constitutive activation and associated proteins involved in cell survival, proliferation, migration, invasion and angiogenesis have led to investigation of NFAT as a drug target (Lu and Huan, 2007, Qin et al., 2014).

Role of NFAT in Regulation of the Immune Response (10 / 160) (p value = 0.005)	
Gene	Gene Function
GSK3B	Glycogen synthase kinase 3 beta. Phosphorylates NFAT promoting nuclear export
GNAZ	Guanine nucleotide binding protein (G protein), alpha z. Associated with Akt signalling.
FYN	Protein-tyrosine kinase oncogene family. It encodes a membrane-associated tyrosine kinase that has been implicated in the control of cell growth.
PPP3R1	Protein phosphate 3, regulatory subunit B alpha. Encodes calcineurin subunit B type 1. Associated with PI3K and MAPK signalling.
ITPR3	Inositol 1, 4, 5 –triphosphate receptor type 3. Mediates the release of intracellular calcium.
GNG11	Guanine nucleotide binding protein (G protein), gamma 11. Regulates cell senescence. Encodes a cell membrane protein involved in transmembrane signalling.
CD79B	CD79b molecule, immunoglobulin-associated beta. Forms part of the B cell receptor. Often mutated in lymphoma
MEF2D	Monocyte enhancer factor 2D. Related to Akt signalling. Role in leukaemogenesis.
MAPK3	Mitogen activated protein kinases/ extracellular signal-regulated kinases (ERKs), act in a signalling cascade that regulates various cellular processes such as proliferation, differentiation, and cell cycle progression in response to a variety of extracellular signals
FCGR3A/FCGR3B	Fc fragment of IgG, low affinity IIIa/b receptor (CD16a/b). Responsible for the removal of antigen-antibody complexes in the circulation. Also expressed on natural killer (NK) cells, FCGR3B is expressed on polymorphonuclear neutrophils.

Table 5.2 Differential expression of genes associated with the role of NFAT in the regulation of the immune response. Upregulated genes are denoted in red and downregulated genes are denoted in green. (Genecards, NCBI Gene, Ingenuity®)

Although the role of NFAT and associated genes have been well characterised in the immune system, their roles are multifaceted. Calcineurin signalling is required for NFAT translocation to the nucleus and due to the weak association of NFAT with DNA, the recruitment of other factors is required for NFAT-mediated gene transcription (Fric et al., 2012, Pan et al., 2013). NFAT isoforms were initially discovered in T-cells, responsible for the transcription of the cytokine interleukin-2 (IL-2) (Pan et al., 2013). Further research led to the discovery that they were in fact responsible for range of cytokines: IL-2, -4, -5, -8, -13, tumour necrosis factor-alpha (TNFα); interferon-gamma IFNγ and granulocyte macrophage colony stimulating factor (GMCSF) (Im and Rao, 2004). In addition to T-cells, NFAT proteins are also activators of mast cells, B-Cells, NK cells, mast cells, megakaryocytes and monocytes (Muller and Rao, 2010).

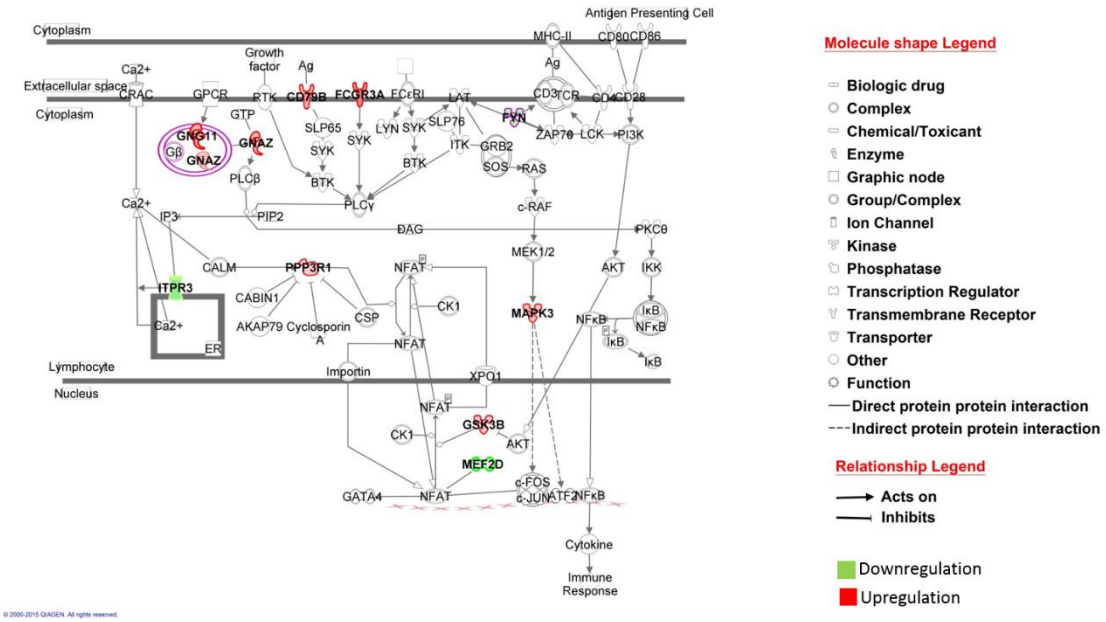


Figure 5.4 The role of NFAT in the immune response was identified as the top altered pathway in NAFLD/NASH-HCC. Red=upregulated, green=downregulated (Ingenuity®, Qiagen).

5.5 Discussion

Firstly, it is important to note that this is pilot data and a larger number of samples would be required in order to confirm differences in genes differentially expressed in this small study. A HCC gene signature that has the ability to detect early-HCC has been developed using similar methods for patients with HCC developed on a HBV aetiology, however this required large numbers of patient samples identify and validate key genes in the development of a gene signature (Shi et al., 2014). That signature remains to be validated and as yet has no clinical utility.

In this preliminary data, there were large differences in the numbers of differentially expressed genes between the three different sample groups. Interestingly, principal component and heat map analyses demonstrated that there were NAFLD/NASH-HCC was more similar to HBV-HCC than it was to NAFLD/NASH, suggesting that there was more variation between the NAFLD/NASH control samples and HCC samples than there was between the two different aetiologies of HCC **Figure 5.2** and **Figure 5.3.**

Investigations into pathway analysis showed that the role of NFAT in the immune response was the top altered pathway. The role of NFAT in the immune response has been well characterised (Fric et al., 2012). NFAT proteins are responsible for the activation of a wide range of immune cells as wells as the expression of multiple cytokines (Im and Rao, 2004, Muller and Rao, 2010). NFAT is a transcription factor that has been linked to various cancer types and it is currently being investigated as a drug target (Lu and Huan, 2007, Pan et al., 2013). There are limited reports of the role of NFAT in HCC. One study showed that the NFATc1 isoform was upregulated in 76 HCC samples by IHC and siRNA knockdown of NFAT led to decreased proliferation in HepG2 cells (Wang et al., 2012a).

Although this data is preliminary, it would be interesting to investigate a larger number of NAFLD/NASH-HCC patients and controls in order to see if these genes were still identified as having a role in the transition from NAFLD/NASH cirrhosis to HCC.

Furthermore, a larger study may identify key genes that could give more insight into the transition from NAFLD/NASH cirrhosis to HCC and lead to the development of a genetic signature that could be utilised as a non-invasive test used to monitor patients with the aim of identifying patients at the early stage of disease progression, rendering them suitable to receive curative treatment.

5.6 Conclusion

- There were 523 differentially expressed genes between NAFLD/NASH controls and NAFLD/NASH-HCC and 604 differentially expressed genes between NAFLD/NASH-HCC and HBV-HCC. Data suggests that there was more variation between NAFLD/NASH controls and NAFLD/NASH-HCC compared to NAFLD/NASH-HCC and HBV-HCC.
- Data in this chapter suggests that the role of NFAT in the immune response is the top altered pathway in PBMCs from NAFLD/NASH -HCC.
- Sample size is small and a large validation cohort would be required to make definite conclusions. Future work required would include increased sample collection to form a training cohort to identify differentially expressed genes that could differentiate between NAFLD/NASH controls and NAFLD/NASH-HCC.

Chapter 6. Summary, final conclusions and further directions

Currently in patients with HCC, liver tissue studies are not routinely employed to guide treatment decisions and serum AFP is the only clinically used tissue biomarker. Limitations associated with the use of AFP include its poor diagnostic sensitivity and specificity, but it is used for monitoring disease progression and for treatment stratification. Alternative biomarkers - that could be clinically useful in terms of diagnosis, prognosis and treatment stratification - are desirable. As liver biopsy is an invasive procedure that carries with it associated risks, including bleeding and tumour cell seeding, low risk and cheap blood-based biomarkers would be particularly attractive. Blood based biomarkers might evaluate serum or plasma proteins, cytokines or cell free DNA. They might also evaluate an aspect of circulating blood cells – either non tumour or tumour cells. So far, no such biomarkers have been clinically validated for use in HCC.

The description of CTCs was reported more than a century ago by Australian physician Thomas Ashworth (Ashworth, 1869). Since then, with the advent of new technologies that are able to detect CTCs more readily and with improved accuracy, further investigations into the clinical utility of CTCs have been performed. The CellSearch system has been clinically validated for use in metastatic breast, prostate and colorectal cancer (Cohen et al., 2008, Cristofanilli et al., 2004, de Bono et al., 2008); however these assays are based on enumeration of epithelial cells alone. It has been considered that characterisation of CTCs may further enhance knowledge of metastasis and hence enhance the clinical utility of CTCs e.g. treatment stratification or prediction of prognosis. The Imagestream has several key benefits over the CellSearch in that cells imaged are of high-resolution; making it possible to discriminate between cells and debris on the basis of morphology using high-quality brightfield images. Furthermore, using the Imagestream meant that it was possible to implement multiple biomarkers into one panel in attempt improve characterisation of CTCs. It was still necessary to ensure that the method developed would be accurate and reproducible as well as practicable for use on clinical samples. Whilst it was possible to run non-enriched samples through the

Imagestream, this was timely, costly and produced vast amounts of data that were not easy to analyse. On the other hand, enrichment techniques often led to poor recovery rates of spiked cells, bias in the cells that were enriched (often on the basis of biomarker expression) or samples that were loaded with debris rendering them unsuitable for Imagestream analysis.

Data presented here previously (**Chapter 2**) demonstrated that enrichment of CTCs could be achieved following red cell lysis, using an immunomagnetic depletion of CD45-positive WBCs, resulting in a ≥95% reduction in haematopoietic cells whilst maintaining a CTC recovery rate of 51.3-65.37%. A further advantage of this method was that it depleted the sample of CD45-positive leucocytes meaning that it was quicker to run and analyse samples but at the same time the depletion method did not positively select for CTCs on the basis of a biomarker, meaning that bias was not introduced into the isolation procedure. Following this CTC enrichment step, samples were rendered suitable for use with the Imagestream; something that had failed by trialling other methods of CTC depletion due to reductions in recovery of spiked cells or production of debris that was imaged by the Imagestream; resulting in unmanageable data files. Heterogeneity in HCC cell lines was observed in initial cell line studies making it an important consideration during the generation of an antibody panel. It was possible to implement a panel of 6 biomarkers (including a DNA dye) without compromising on sensitivity. Furthermore, this method was found to be practical from a clinical perspective and reproducible for the isolation of CTCs in other cancer types including ovarian, oesophageal and thyroid (Dent et al., 2015).

Following optimisation of a method for CTC isolation and detection using the Imagestream, blood samples from an HCC patient cohort were tested for the presence of CTCs using a biomarker panel consisting of EpCAM, CK, AFP, DNA-PK, GPC3 and SULF2 (**Chapter 3**). This patient cohort consisted of patients with varying aetiology and stage of disease and samples were obtained at different points throughout their clinical management. Between 1 and 1642 CTCs were detected in 45/69 (65%) of HCC patients compared to 0 CTCs detected in cirrhotic or healthy control samples. In this study,

observers were not blinded to patient status – a potential source of bias – which could be overcome by getting additional observers to review the data files in a blind manner. The presence of ≥1 CTCs/4ml indicated a remarkably worse prognosis in terms of median OS (24.5 months) compared to CTC-negative samples (12 months), Kaplan-Meier with Log-Rank test $p<0.0001$. In patients with ≥1 CTC/4 ml, TTP was also shorter as well using Kaplan-Meier survival analysis with Log-Rank test $p=0.006$. The percentage of HCC patients with CTCs detected was higher in this cohort than in other CTC studies in HCC, attributed to the inclusion of biomarkers in addition to CK and EpCAM (Morris et al., 2014, Schulze et al., 2013). Of the biomarkers that were included in the panel, the epithelial biomarker pan-CK was the most commonly expressed (in 29% of CTC-positive samples). Of the exploratory biomarkers, DNA-PK was the most commonly detected (in 24% of CTC-positive samples). In a smaller separate cohort of patients (n=14), CTC expression of two further biomarkers were tested: pERK as a surrogate marker of Ras/raf/MAPK pathway activation and c-Met, which may identify patients that would respond to the c-Met inhibitor tivantinib (Newell et al., 2009, Santoro et al., 2013a, Santoro et al., 2013b). Whilst, the presence of ≥1 CTC/4ml may be used to provide prognostic information, biomarkers that would be able to stratify patients for therapy could be highly advantageous to the clinical management of HCC. CTCs are a possible source to investigate such markers, since it is hypothesised that CTCs are responsible for disease recurrence; although it is important to note that HCC tumour heterogeneity and the metastatic potential of CTCs should be taken into consideration. One of the limitations of this study is that samples taken from patients post-treatment were not taken at defined time points. Previous CTC studies have measured CTC counts prior to treatment and at specified time points post-treatment; concluding that patients whose CTC counts declined post-treatment had a better prognosis (Cristofanilli et al., 2004, de Bono et al., 2008, Cohen et al., 2008). It is possible that in patients where CTCs were measured soon after treatment that there was a surge in CTCs but, time permitting, it would have been interesting to follow up these patients long term, taking repeated CTC measurements at various time points following their treatment and explore survival in this group.

It was also possible to identify biomarker-negative cells on the basis of size and DNA content, which discriminate CTCs from WBCs (**Chapter 2-3**). Biomarker-negative cells detected correlated with poor HCC prognosticators including tumour size, encephalopathy and inversely with serum albumin. It is possible that these cells expressed biomarkers not included in the panel, potentially mesenchymal markers. Some methods of CTC isolation or detection are size-dependent (e.g. filtrations methods such as the ISET); however it was not known at the time whether or not HCC CTCs would be larger in size compared to other haematopoietic cells. Further objects identified included clusters of CTCs and the interaction of leucocytes with CTCs although the clinical significance of this is not yet known. It may be suggestive of CTCs being recognised by leucocytes for their destruction or that CTCs are travelling in aggregates with CTCs as a protection mechanism against immune destruction.

In addition to Imagestream CTC exploration, a pilot study to see whether or not ctDNA might be a potential source of tumour-derived information that could be detected using standard biobanked blood samples from patients with HCC. Initial attempts focused on KRAS mutational status, based on evidence of RAS signalling pathway activation in HCC (Newell et al., 2009) and the postulation that the frequency of KRAS mutations may be increased in patients with HCC developed on a background of NAFLD/NASH (Whitehead et al., 2015). Whilst it was possible to isolate ctDNA from frozen HCC plasma samples, frequency of KRAS mutations was low (~5%). Results may be improved by using a pre-enrichment step, using a ctDNA detection technology with higher sensitivity, although it is possible that KRAS mutations in NAFLD HCC are not as prevalent as hypothesised. An attempt to detect TERT promoter mutations in ctDNA was unsuccessful employing similar methods. The GC rich content of the TERT promoter region proved to be a barrier to suitable primer design.

HCC – along with other types of cancer - is a disease that commonly arises on a background of inflammation (Nikolaou et al., 2013). While the role of the tumour microenvironment in HCC remains to be fully understood, it is considered that tumour-associated inflammation is tumourigenic (Balkwill et al., 2012, Hanahan and Coussens,

2012, Hanahan and Weinberg, 2011). It has been previously suggested that counts or ratios of circulating immune cells may be of clinical value in terms of prognosis (Dan et al., 2013, Xu et al., 2014, Gomez et al., 2008).

As part of this work, data was retrospectively collected from a cohort of 585 Newcastle HCC patients and examined for correlations between circulating immune cell counts and other HCC prognostic biomarkers. Associations with HCC stage (TNM and BCLC) as well as assessment of underlying liver function (Child-Pugh) and ECOG performance status were explored. Low platelet counts have been associated with cirrhosis and worsening CLD (Qamar et al., 2009, Afdhal et al., 2008) and this was reflected in this data set with platelet counts decreasing with worsening Child-Pugh score. However, platelet counts increased with increasing TNM stage and BCLC stage; indicating that increasing tumour size and spread is associated with more tumour-associated inflammation and increased platelet counts (**Chapter 4**). The association between high platelet counts (thrombocytosis) and worse survival has been documented in solid tumours (Lin et al., 2014). Studies have indicated that tumour cells are able to activate platelets resulting in tumour associated inflammation, proliferation and angiogenesis; promoting tumour growth and metastasis (Lin et al., 2014, Sharma et al., 2014, Gay and Felding-Habermann, 2011). In multivariate Cox analyses, neutrophils were found to be independent prognostic indicators. Such relationships and significance of these measures were also observed in a cohort of HCC patients managed in Hong Kong (**Chapter 4**). Whilst above median counts of neutrophils were identified as being a significant poor prognostic indicator in this large cohort of HCC patients, the phenotype of neutrophils (N1 vs N2) may be of further importance since research has identified N2 neutrophils as tumourigenic. The role of TGF-β as an effector molecule for the switch from an anti-tumourigenic (N1) phenotype to a pro-tumourigenic (N2) phenotype may require further investigation in this context, since TGF-β has already been identified as a therapeutic target in HCC and inhibitors against TGF-β are being trialled (Giannelli et al., 2011, Giannelli et al., 2014). In the combined cohort, high counts of neutrophils alone and the NLR and SII were associated with significant differences in median OS in patients receiving non-surgical treatment.

It has already been noted that the use of the TNM staging system alone in HCC is limited due to the absence of inclusion of assessment of the underlying liver disease. On the other hand, scoring systems that have been implemented for use in HCC often fail to take into account characteristics of the tumour **Table 1.1**. The BCLC staging system addresses these limitations by including both the assessment of underlying liver disease and tumour characteristics to stratify patients for treatment. However, there is currently no scoring system for use in HCC that includes a measurement of tumour-associated inflammation. The SII provides a marker of systemic inflammation though it is limited since it may not be representative of the tumour microenvironment and does not include tumour parameters; although it may be useful in adjunction with other scoring systems such as the BCLC staging system. In BCLC-C patients, an above or below median SII was able to differentiate between patients with a better or worse median survival **Table 4.25** although the SII did not remain significant in multivariate analysis in this patient group. The SII may help to determine whether or not treatment would provide a survival benefit in these patients although this would need to be explored in a larger cohort of BCLC-C patients receiving the various treatment modalities.

Additional work was performed as part of a collaborative study with Professor Kam Hui's research group at the National Cancer Centre Singapore (**Chapter 5**). Professor Hui's research group had previously identified a genetic signature in circulating PBMCs in HCC patients; demonstrating that three differentially expressed genes - CXCR2, CCR2 and EP400 – were able to detect early HBV associated HCC irrespective of AFP level, distinguishing HCC patients from healthy people and control patients with HBV (Shi et al., 2014). This study was pertinent, given our data implicating counts of circulating inflammatory cells – particularly neutrophils as the most abundant PBMC – as independent predictors of poorer prognosis (**Chapter 4**); Due to the increasing prevalence of patients with HCC developed on a background of NAFLD/NASH, a pilot study based on a small cohort of HCC patients with this aetiology were selected along with NAFLD/NASH controls. Differential gene expression of PBMCs from these two groups was assessed and also compared with HCC patients with a HBV/HCV aetiology. It was found that in terms of differentially expressed genes, the NAFLD/NASH-HCC samples were more similar the HBV/HCC samples than the NAFLD/NASH controls.

Although this study was small and confirmation in a larger cohort is needed, the NFAT pathway was identified as the most altered one in NAFLD/NASH HCC patients. The NFATc isoform has previously been identified as upregulated in HCC, with its knockdown resulting in the decreased proliferation of HepG2 cells (Wang et al., 2012a). Furthermore, NFAT has been implicated in other cancers, with its overexpression, constitutive activation and associated proteins being implicated in tumorigenicity and metastatic spread of disease - resulting in its pursuit as a candidate therapeutic target (Macian, 2005, Pan et al., 2013, Lu and Huan, 2007, Qin et al., 2014).

6.1 Further Directions

- Increased blood sample collection from HCC patients to assess for the presence of CTCs and explore the utility of biomarkers trialled in a small cohort of patients i.e. c-Met and pERK.
- Concurrent CTC isolation using the DEParray or size-dependent isolation methods i.e. ISET would enable downstream characterisation of HCC CTCs which may lead to the identification of targetable key genetic drivers in populations of cells capable of initiating recurrence of disease.
- Assessment of the phenotype of neutrophils in patients with HCC to determine whether N1 or N2 neutrophils predominate.
- Exploration of gene signatures in PBMCs in a larger cohort of patients with NAFLD/NASH-HCC aetiology in order to determine key genetic alterations and validate these findings.

Appendix

Published Papers

OGLE, L. F., ORR, J. G., WILLOUGHBY, C. E., HUTTON, C., MCPHERSON, S., PLUMMER, R., BODDY, A. V., CURTIN, N. J., JAMIESON, D. & REEVES, H. L. 2016. Imagestream detection and characterisation of circulating tumour cells - A liquid biopsy for hepatocellular carcinoma? J Hepatol, 65, 305-13.

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Conferences – Awards

Junior Investigator Award. The International Liver Cancer Association 9th Annual Conference, 05/09/2015.

Top-scored poster. The International Liver Cancer Association 9th Annual Conference, 05/09/2015.

Young Investigator's Bursary. The International Liver Congress 2014, 50th annual meeting of the European Association for the Study of the Liver, 23/04/2015.

1st prize poster award. Northern Institute for Cancer Research and Institute of Genetic Medicine Joint Research Day, 11/12/2014.

Young Investigator's Bursary. The International Liver Congress 2014, 49th annual meeting of the European Association for the Study of the Liver, 12/04/2014.

BASL Travel Award. British Association for the Study of the Liver Annual Conference, 04/09/2013.

Conferences – Oral Presentations

OGLE, L. F., STOCKEN, D., PLUMMER, E. R., CURTIN, N. J., JAMIESON, D. & REEVES, H. L. 2015. Liquid Biopsy to Detect and Characterise Circulating Tumour Cells in Patients with Hepatocellular Carcinoma Using High Resolution Imagestream Multichannel Fluorescent Microscopy. The International Liver Cancer Association 9th Annual Conference. Paris.

OGLE, L. F., JAMIESON, D., O'DONNELL, R., DENT, B., CURTIN, N. J., BODDY, A. V., PLUMMER, E. R. & REEVES, H. L. 2014. Detection of circulating tumour cells (CTCs) in hepatocellular (HCC) cancer patients using the ImageStream^{X}. The International Liver Congress 2014, 49th annual meeting of the European Association for the Study of the Liver. London.

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