THE EXPRESSION OF CD73 IN HEPATOCELLULAR CARCINOMA AND ITS MICROENVIRONMENT IN THE CONTEXT OF CHRONIC INFLAMMATION

by

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Abstract

Introduction

Primary liver cancer, hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide. HCC often develops in the context of liver fibrosis which contributes to an immunosuppressive tumour microenvironment (TME). Overcoming the TME in HCC is a major challenge to successful therapy. Better understanding of the cell specific contribution to immunosuppression in the TME is required to help boost current immunotherapy. Hepatic sinusoidal endothelial cells (HSEC) are the gatekeeper for immune cell recruitment and pilot RNA sequencing data showed significant upregulation of CD73 in tumour endothelium. CD73 exerts an immunosuppressive effect through production of adenosine in the extracellular space. We sought to understand its expression in HCC and potential role in HSEC.

Methods

Immunohistochemistry for CD73 was performed on both normal liver (NL) and a range of chronic liver diseases (CLD) as well as a cohort of 99 HCC samples. Qualitative and quantitative analysis was performed and results compared with clinical data for the HCC cohort. Quantitative RT-PCR for canonical CD73 and a spliced variant was undertaken on whole liver tissue and hepatocyte cell lines Huh7 and HepG2. Primary HSEC isolated from liver tissue using a magnetic bead technique were analysed for CD73 expression by immunofluorescence and RT-PCR. CD73 expression was also studied in HSEC in static conditions and compared to HSEC that had been subject to shear stress.

Results

Immunohistochemical staining was consistent with CD73 expression on endothelial and epithelial cells in normal liver and CLD. Staining of the canaliculi was noted to be more pronounced in biliary disease compared to NL. The expression pattern of CD73 in HCC was heterogeneous, with a variation between membranous and cytoplasmic expression on

tumour hepatocytes. Membranous expression was associated with a trend towards increased incidence of vascular invasion, a marker of poor prognosis, and worse overall survival, although this did not reach statistical significance. In contrast, peri-tumour vasculature was positive for CD73 in all cases of HCC in the cohort.

At the transcript level, the spliced variant of CD73 (CD73S) was minimally expressed in NL tissue, but increased in CLD tissue, particularly biliary disease.

Isolated HSEC maintained expression of CD73 in culture and gene expression was detected in both HSEC and the hepatocyte cell lines, Hep G2 and Huh-7. The spliced variant of CD73 was significantly higher in Huh-7 than HSEC. There was no difference in gene expression due to shear stress.

Conclusion

I found that CD73 is expressed on the sinusoids and canalicular structures of both normal liver and chronic inflammatory disease. CD73 expression was variable in HCC, with membranous and cytoplasmic staining in tumour cells. In all cases of HCC, the peritumoural vasculature was positive for CD73, which may explain the upregulation of CD73 in previous RNAseq studies of tumour versus non-tumour endothelium. HSEC maintain their expression of CD73 in culture. The CD73 spliced variant is significantly upregulated in the hepatoma cell line Huh 7 at the transcript level, and may reflect the cytoplasmic staining of CD73 on IHC that was unique to HCC tumours. This work highlights the need for further studies into the function of CD73 in HSEC especially around immune cell activation and recruitment to the TME of HCC and its future potential as a vascular therapeutic target.

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Abbreviations

AMP	Adenosine monophosphate
AML	Acute myeloid leukaemia
ASIR	Age standardised incidence rate
ATP	Adenosine triphosphate
CD	Cluster of differentiation
cDNA	complementary Deoxyribonucleic acid
CI	Checkpoint inhibitor
CLR	Centre for Liver Research
DAB	3, 3’-Diaminobenzidine
DAMPs	Damage associated molecular patterns
DAPI	4', 6-Diamidino-2-Phenylindole, Dihydrochloride
DBD	Donor after brainstem death
DC	Dendritic cell
DCD	Donor after cardiac death
EGFR	Epidermal growth factor receptor
EMT	Endothelial to mesenchymal transition
GDP	Gross domestic product
GPI	Glycosylphosphatidylinositol

HCC	Hepatocellular carcinoma
HIF-1	Hypoxia inducible factor 1
HRP	Horseradish peroxidase
HSC	Hepatic stellate cell
HSEC	Hepatic sinusoidal endothelial cell
IFN-γ	Interferon gamma
IL	Interleukin
IMC	Isotype matched control
KC	Kupffer cell
LPS	Lipopolysaccharide
MHC-I and -II	Major histocompatibility complex I and II
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PRR	Pattern recognition receptor
RT	Room temperature

SABR	Stereotactic ablative radiotherapy
SR	Scavenger receptor
TACE	Trans-arterial chemoembolisation
TGFβ	Transforming growth factor beta
TLR	Toll like receptor
TNFα	Tissue necrosis factor alpha
Tregs	Regulatory T cells
UK	United Kingdom
VEGF	Vascular endothelial growth factor

Chapter 1

INTRODUCTION

1.1 Hepatocellular carcinoma is a global disease

The majority of cases of primary liver cancer (hepatocellular carcinoma, HCC) arise due to underlying chronic liver disease, such as chronic viral hepatitis or non-alcoholic steatohepatitis (NASH). Any cause of progressive liver inflammation leading to end stage fibrosis, termed cirrhosis, increases the risk of HCC. Each aetiological factor has varying prevalence across the world, but in combination they drive this global health problem, making HCC the third leading cause of cancer mortality worldwide.[1]

Chronic hepatitis C affects approximately 71 million people - 1% of the global population - and prevalence is greatest in Europe and the East Mediterranean.[2] [3] Chronic hepatitis B is endemic in China and sub-Saharan Africa, where prevalence can exceed 20%.[4] Efforts to control viral hepatitis through direct acting antivirals and steps to limit mother to child transmission have borne moderate results, such as the infant vaccination programme against hepatitis B in Taiwan, which has significantly reduced the incidence of HCC among young adults.[5] However, most countries remain a considerable distance from meeting the World Health Organisation's ambitious commitment to reduce new viral hepatitis infections by 90% by 2030.[2]

Other factors which impact geographic variation of HCC include the fungal carcinogen aflatoxin B1, which grows in crops used for animal feed and human consumption, and is also endemic to South East Asia and Africa.[6] In contrast in the West, alcohol remains the commonest cause of liver cirrhosis, and consumption is highest in Europe and the Americas.[7]

Cirrhosis may also develop due to autoimmune liver disease: primary biliary cholangitis, primary sclerosing cholangitis or autoimmune hepatitis. Less commonly, cirrhosis may also arise due to genetic conditions such as alpha-1 antitrypsin deficiency and hereditary haemochromatosis. The latter causes an excess absorption of iron from the gastrointestinal tract, which is deposited in the liver. Although rare, it is one of the most carcinogenic liver diseases.[8]

In addition to these causes of liver disease, the incidence trajectory of non-alcoholic fatty liver disease (NAFLD) is set to change the landscape of HCC for the worse. NAFLD is associated with the metabolic syndrome of type II diabetes mellitus, hypertension and hyperlipidaemia. All are influenced, to some degree, by obesity. Often described as a pandemic, obesity continues to increase across both low- and high-income countries. One in four adults in England is now classed as obese, in Argentina and the United States it is one in three.[9] As a result, global prevalence of NAFLD is around 25%.[10] Approximately one fifth of those with fatty liver will develop inflammation and fibrosis causing non-alcoholic steatohepatitis (NASH). NASH is a significant risk factor for developing liver cancer, as reflected in a recent meta-analysis showing the global incidence of HCC in NASH is more than 10 times that of NAFLD (5.29 per 1000 person-years vs 0.44 per 1000 person-years)[10]. As with chronic viral hepatitis, HCC can develop in NASH in the absence of cirrhosis, and therefore may arise before individuals have developed symptoms of chronic liver disease, received a diagnosis, or qualify for routine HCC surveillance.[11] [12] Although chronic hepatitis C and haemochromatosis are the most carcinogenic liver diseases, the endemic nature of hepatitis B and increasingly, of NAFLD, mean that these two diseases are the

commonest causes of HCC worldwide. As the obesity prevalence grows, so too will NASH. Therefore HCC incidence is set to increase globally over the coming decades.

The wide range of aetiological factors for HCC in different environments and people groups means that it carries a high burden of morbidity and mortality across all continents (Figure 1-1). We therefore urgently need to better understand the oncogenesis of liver cancer, in order to improve treatment and survival.

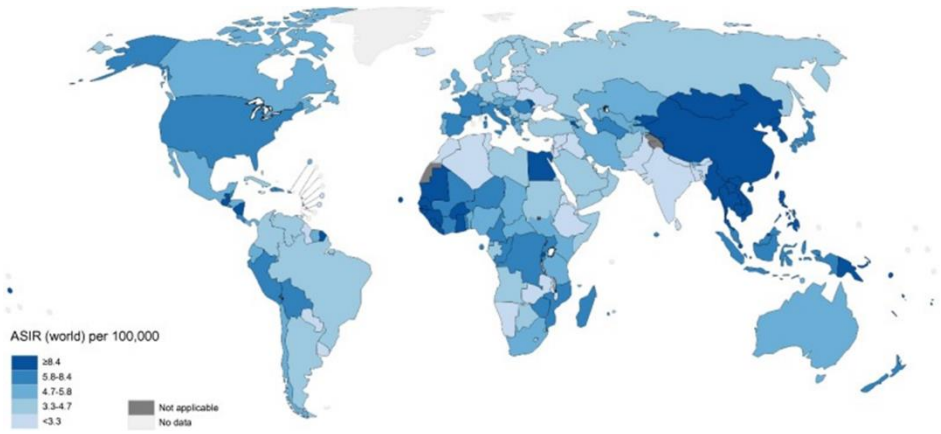


Figure 1-1 The incidence of HCC in adult males worldwide

Graphic reproduced from Singal et al.[13] The highest incidence is seen in China and West Africa where hepatitis B is endemic, followed by Europe and North America where NASH and ArLD predominate. ASIR (Age Standardised Incidence Rate).

1.2 Survival in HCC

Not only is liver cancer common, it is difficult to cure. HCC is the sixth most prevalent cancer in the world, yet the third leading cause of cancer death. Poor survival is observed across both low and high resource countries. For example, the average five-year survival for primary liver cancer is 8% in both Finland (GDP $48, 771 per capita) and Mongolia (GDP $4,

339 per capita) [14] [15]. Although this is a crude assessment of health economics, it serves to demonstrate that outcomes in HCC are determined to a greater extent by tumour biology and the dearth of effective treatments rather than the availability or quality of healthcare. This is also shown when comparing survival in HCC with other types of cancer. The majority of cancers in England have a five-year survival greater than 50%, but for liver cancer it is just 13% (Figure 1-2).[16]

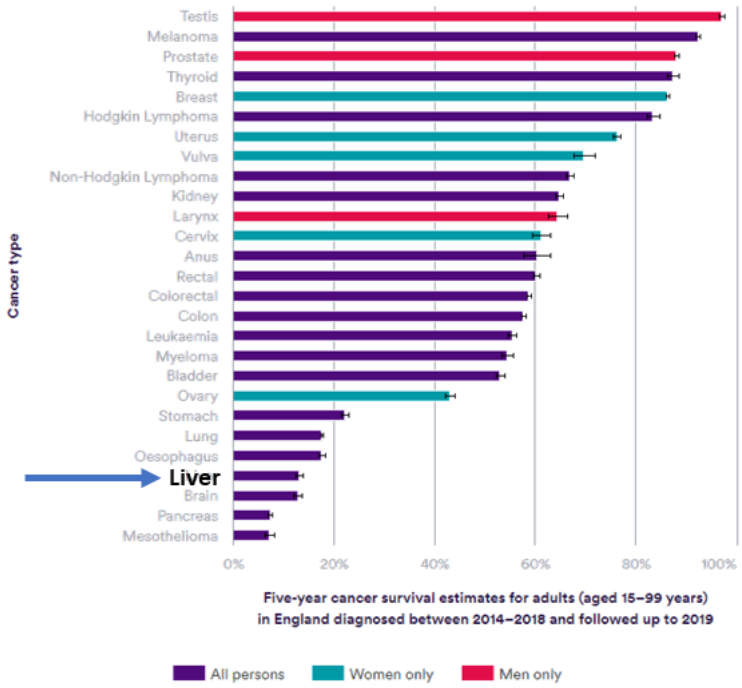


Figure 1-2 Five-year cancer survival for adults in England diagnosed between 2014 and 2018.
Based on graphic created by the Nuffield Trust.[16]

There are a number of factors contributing to the significant mortality rates in hepatocellular carcinoma. Firstly, symptoms of liver cancer are non-specific, and usually do not develop until later in the disease course. Thus, unlike testicular or breast cancer for example, patients with HCC are less likely to present in the early stage of disease, when treatment is more likely to be curative. Therefore, six monthly screening for liver tumours using ultrasound

imaging and measurement of the serum tumour marker alpha-fetoprotein is recommended for all patients with cirrhosis in the UK. However, there is insufficient evidence to support the cost-efficacy of screening non-cirrhotic patient groups at risk for HCC, and therefore patients with chronic hepatitis B, hereditary haemochromatosis or NASH are not routinely included in HCC surveillance.[12]

Secondly, as the majority of cases of HCC develop in the context of cirrhosis, the risk of precipitating liver failure due to cancer therapy in a patient with underlying liver disease can significantly limit the range of treatment which can be offered. Furthermore, patients who have developed one liver tumour are at higher risk of developing further HCC's. For example, five year recurrence rates are 70% following resection of HCC.[17] Therefore, unlike other cancers, successful treatment of an HCC is less likely to lead to long term remission.

Finally, immunotherapy has revolutionised the prognosis of some cancers such as non-small cell lung cancer and melanoma. Recent studies in HCC have shown promising results, but there remain a significant proportion of patients who do not respond to treatment.[18] Possible reasons for this will be discussed below.

1.3 Current therapeutic options for HCC

Treatment of HCC can be broadly classified into potentially curative modalities using ablation or surgery, and non-curative options using embolization, radiotherapy or systemic therapy.

Treatment decisions are guided by the Barcelona Clinic Liver Cancer (BCLC) staging system (Figure 1-3)

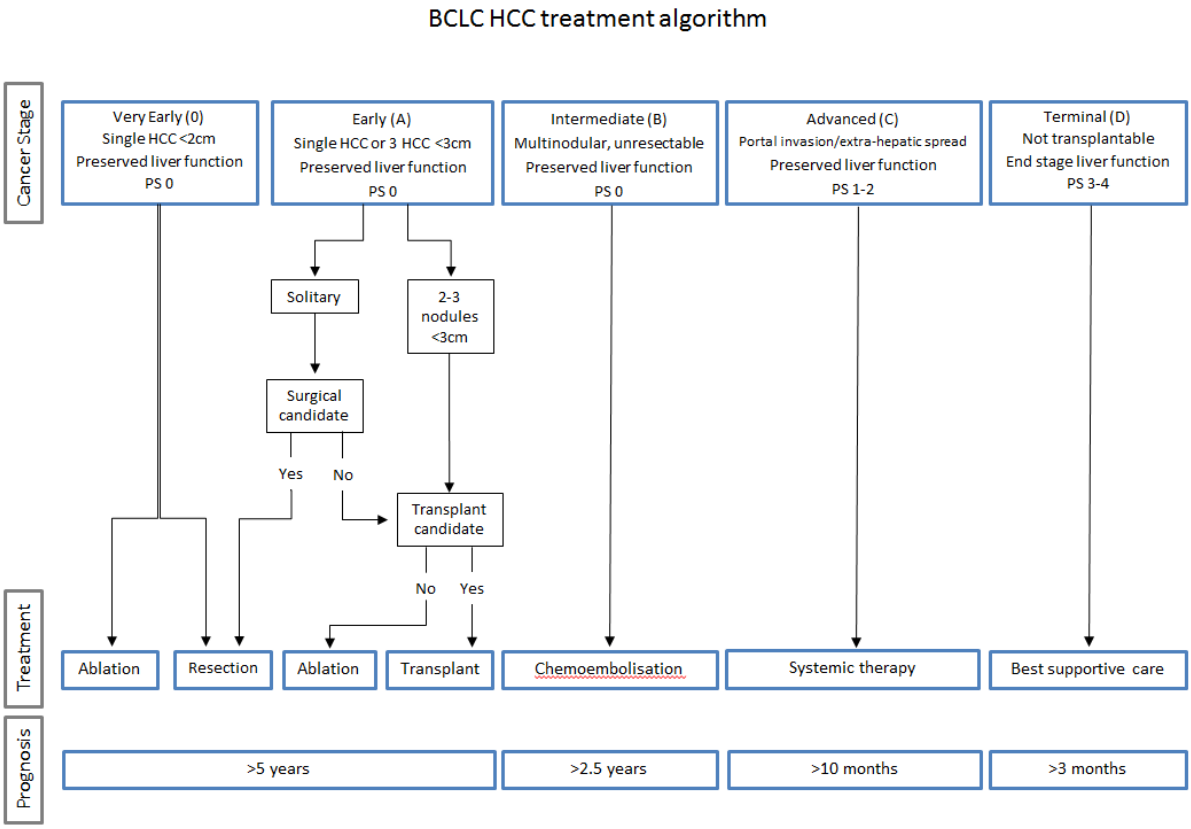


Figure 1-3 Barcelona Clinic Liver Cancer (BCLC) staging system.

Adapted from EASL Clinical Practice Guidelines.[12] PS (Performance Status) where 0 = fully active, 1-2 = unable to perform strenuous activity, but ambulatory and self-caring, 3 = capable of limited self-care, 4 = bedbound and fully disabled.

1.3.1 Ablation, surgical resection and liver transplantation

Small tumours <2.5cm may be treated with ablation or surgical resection. These treatments may be curative, although patients with predisposing risk factors will remain vulnerable to developing further HCC's. Those with advanced cirrhosis are often at an increased risk of liver failure after surgical resection, and therefore this option may only be offered to select patients.

Liver transplantation yields the best outcomes in HCC, both in overall survival and disease-free survival. The median five year survival post-transplant for HCC is 65% in cirrhotic patients, and slightly lower in non-cirrhotic cases (56%).[19] This is significantly better than other treatment modalities. However, specific criteria must be met to qualify for liver transplantation, both in respect to tumour burden and cancer stage, and also general fitness and co-morbidities.[20] Once accepted on the waiting list, a number of patients will progress outside of transplant criteria and have to be removed from the list, or die before receiving a transplant.

1.3.2 Embolisation and radiotherapy

Trans-arterial chemoembolisation (TACE) directly targets the tumour by disrupting its blood supply, with or without deposition of drug-eluting beads that release chemotherapy agents to augment tumour necrosis (DEB-TACE). This treatment may be used as a bridge to transplantation, or as a non-curative treatment. With the latter, five-year survival is reported between 8 and 18.5%.[21-23]

Stereotactic ablative radiotherapy (SABR) may be used alone or in combination with other treatment modalities. It is a useful option for those who might not tolerate TACE, or where TACE is technically challenging.

1.3.3 Systemic therapy

The principle systemic treatment for HCC has been the tyrosine kinase inhibitor sorafenib. This palliative therapy extends life expectancy by an average of three months.[24] More recently, second line options lenvatinib and regorafenib have been developed.

Checkpoint inhibitors (CI's) are a class of immunotherapy agents that have transformed the prognosis of some cancers, such as melanoma and non-small cell lung cancer.[25] [26] A monoclonal antibody targets PD-1 receptors on T cells, or the ligand (PD-L1) on tumour cells that, when activated, leads to inhibition of T cell activation, or premature T cell death. In HCC, combination therapy with the CI atezolizumab and VEGF inhibitor bevacizumab has shown superiority to sorafenib.[27] However, as with many treatments for HCC, survival outcomes are measured in months rather than years. The limited response of liver cancer to immunotherapy leads us to question what is unique within the liver microenvironment, and how it affects oncogenesis and the immune response.

1.4 The Liver Microenvironment

1.4.1 Basic Liver Anatomy and Blood Supply

The liver is the largest solid organ in the body and essential for life. Liver parenchyma is formed of hepatocytes and cholangiocytes. Hepatocytes are the primary epithelial cells of the liver and perform the majority of its functions including carbohydrate metabolism, detoxification and production of cholesterol, protein and bile salts. Cholangiocytes line bile ducts and modify bile composition. Hepatocytes are organised into hexagonal structures

with a central vein called lobules.[28] Adjacent to each corner of a liver lobule is a portal tract, comprised of a hepatic artery, portal vein and bile duct (Figure 1-4). Blood from the artery and portal vein combines to flow across the lobule through sinusoids, specialised low shear channels that perfuse hepatocytes, before draining into a central vein. This dual blood supply is a unique feature of the liver, with the majority of hepatic blood flow – approximately 75% - being supplied by the portal vein, delivering partially oxygenated, nutrient rich blood from the gastrointestinal tract, gallbladder, pancreas and spleen.[29]

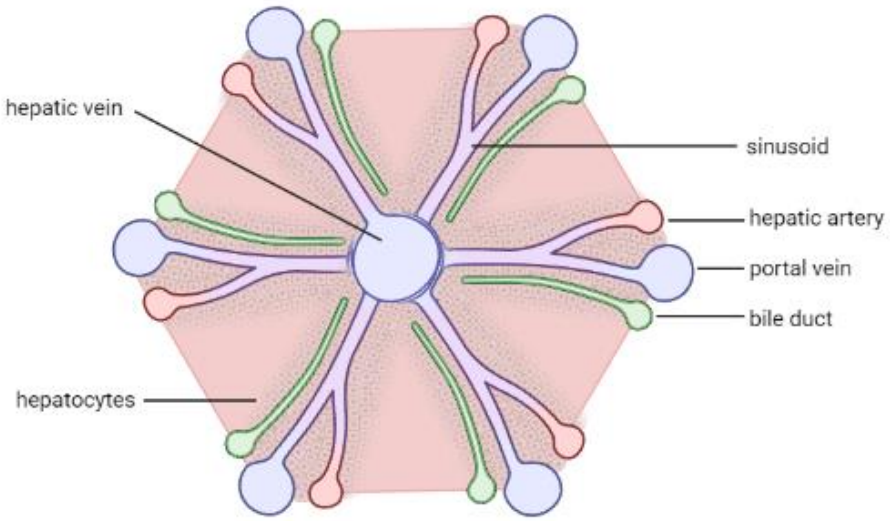


Figure 1-4 A liver lobule.

A bile duct sits adjacent to branches of the hepatic artery and portal vein to form a portal tract, or triad. Arterial and venous blood flows through sinusoids before draining into a central vein, which ultimately drains into the hepatic vein and the inferior vena cava. Original figure created using Biorender.

Sinusoids are lined by hepatic sinusoidal endothelial cells (HSEC), a group of highly specialised endothelia characterised by increased permeability due to fenestrations, open pores in the cell membrane and cytoplasm, and the absence of a basement membrane and

tight junctions.[30] Fenestrations allow passive diffusion of particles, solutes and fluid from blood to the liver parenchyma,[31] thereby facilitating key functions of the liver, such as the absorption and processing of glucose, protein and lipids for energy storage, and the clearance of waste products from the blood.

Unlike other vessels, sinusoids lack a smooth muscle layer to adjust the diameter of the lumen and control flow. There is evidence that nitrous oxide and other vasoactive substances secreted by HSEC are the primary means of regulating blood flow through sinusoids to maintain flow at low shear stress despite fluctuations in portal blood volume.[32]

Liver lobule anatomy can be further characterised into three functional zones determined by the proximity of HSEC and hepatocytes to blood flow. Zone 1 is periportal, adjacent to the portal triad where concentrations of oxygen and nutrients are highest. This is followed by a transition zone (zone 2), then the third, or peri-central, zone surrounding the central vein, where glycogenesis, lipogenesis and clearance of circulating ammonia is performed.[33]

Non-parenchymal cells of the liver include hepatic stellate cells (HSC), which are found between HSEC and hepatocytes in the space of Disse. In the quiescent state their principal function is to store vitamin A. They may be activated by a range of factors and immune cells, including HSEC, Kupffer cells or damage associated molecular patterns (DAMPs) released from injured hepatocytes.[34] Transforming growth factor β (TGFβ) from macrophages

induces HSC's to transdifferentiate into myofibroblasts that secrete extracellular matrix, such as collagen, which is deposited in the extracellular space leading to liver fibrosis.[35] [36] HSCs can also adopt roles of phagocytosis and antigen presentation.[37] [38]

Other innate immune cells such as natural killer cells, dendritic cells and Kupffer cells, the liver resident macrophages, are situated within sinusoids. HSEC interact with innate and adaptive immune cells to directly influence liver immunity, as discussed below.

1.4.2 Hepatic Sinusoidal Endothelium and Immune Tolerance

In addition to nutrients, the portal vein is also a conduit for food antigens and bacteria from the gut microbiome, exposing the liver to PAMPs (pathogen associated molecular patterns) such as lipopolysaccharides (LPS) from cell walls of gram negative bacteria, as well as DAMPs from autologous cells that are injured or dying.[39] [40] In order to withstand the high exposure to foreign antigens, the liver fosters a relatively immunotolerant state compared to other organs. It is able to detect and clear antigens from the circulation without activating a systemic inflammatory response. One of the principal ways immune tolerance is maintained is via the sinusoids.

HSEC facilitate the clearance of PAMPs from the systemic circulation. They express a range of pattern recognition receptors (PRR's), further categorised into toll like receptors (TLR's) and scavenger receptors, that promote endocytosis of a range of ligands including LPS, viruses and lipids.[41] Both HSEC and Kupffer cells show tolerance to repeated LPS

exposure. For example, HSEC are able to respond to LPS via TLR4 without triggering significant leucocyte adhesion.[42] [43] Furthermore, HSEC control the location of KC within the liver, promoting KC positioning in the periportal zone under LPS exposure from the gut.[44]

In respect to the adaptive immune system, sinusoidal endothelia play a dynamic role in lymphocyte activation and recruitment through chemokines and antigen presentation. Adhesion molecules and chemokines on the HSEC luminal surface, such as CXCL12 and CXCL9, facilitate binding of circulating T cells and their migration across HSEC into the space of Disse via a paracellular or transcellular route, or by migrating into and across adjacent HSEC, termed intracellular crawling (Figure 1-5).[45] [46] In inflammatory conditions, HSEC increase the expression of adhesion molecules VCAM-1, ICAM-1, stabilin-1 and CD31, thereby acting in a dynamic way to promote T cell adhesion and recruitment.[30] [47]

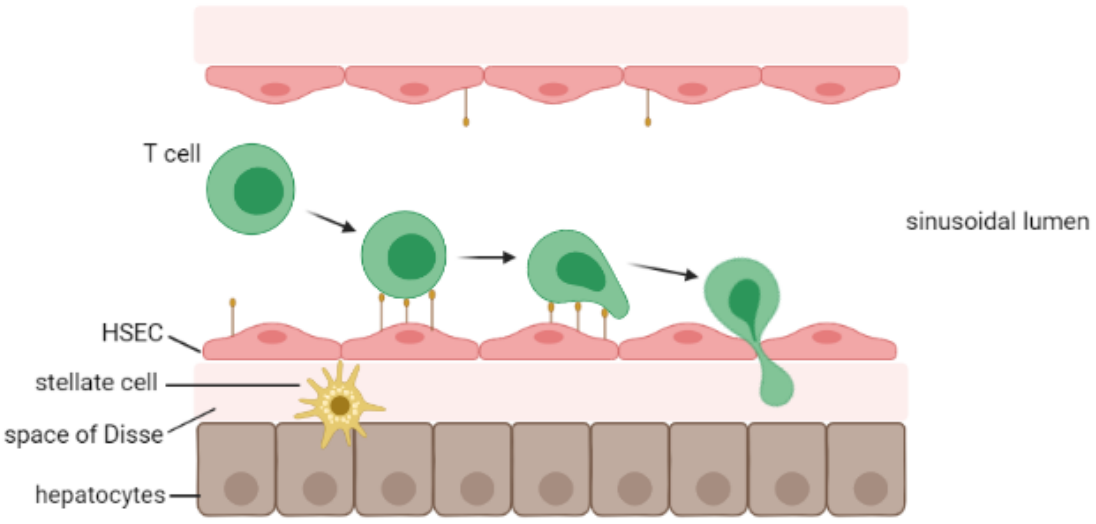


Figure 1-5 Adhesion and migration of circulating T cells across hepatic sinusoidal endothelial cells (HSEC). Original figure created using Biorender.

HSEC also express major histocompatibility class I and II (MHC-I and -II).[46] HSEC present antigens to naïve $CD8^+$ T cells via MHC-I. Once the T cells have been activated, HSEC subsequently express PDL-1 (programmed cell death 1), a co-inhibitory protein that limits T cell expansion, thereby moderating the immune response and contributing to T cell tolerance.[48]

The hepatic ability to execute an immune response within the confines of the liver is crucial to prevent unnecessary and overwhelming inflammation despite constant exposure to foreign antigens. However, hepatic tolerance may be exploited by cancer cells to evade immune-mediated apoptosis, thereby enabling the growth and development of malignant tumours.

1.4.3 The tumour microenvironment

During oncogenesis, cells overcome and evade usual restrictions on growth and division, and by doing so, realise malignant potential. Cancer cells interact with their surroundings in an abnormal way, including invasion of other tissues, dysregulated angiogenesis and aberrant interactions with the immune system. Under normal physiological conditions, the effects of immune cell activation are regulated to prevent collateral damage to healthy tissue. Cancer cells may use such mechanisms to inappropriately limit the immune response to the tumour, hence producing an immune-permissive tumour microenvironment.

The Shetty group has been studying the microenvironment in HCC, focusing on endothelial cells. Pilot work has identified the receptor CD73 as a protein of interest (unpublished). RNA sequencing was performed on endothelium isolated from primary liver tumours and matched non-tumour endothelial cells. Gene ontology analysis of the data demonstrated that the most significantly differentially regulated pathways are all immune related, with profound suppression of immune activating pathways in tumour endothelium. Interestingly this was associated with a significant upregulation of CD73 at the transcript level (gene NT5E), leading me to study its role in the liver microenvironment (Figure 1-6).

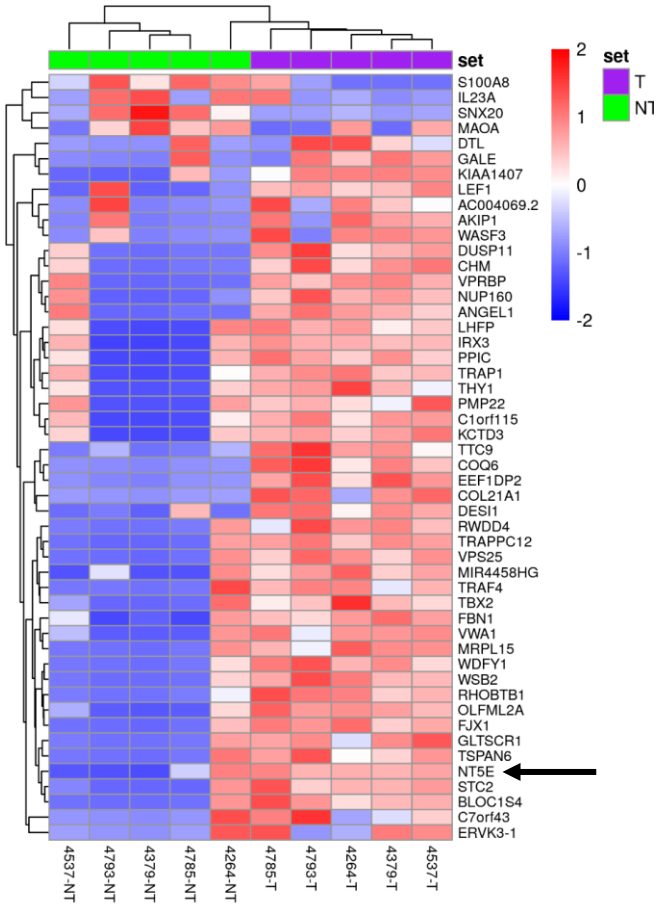


Figure 1-6 Heatmap of RNA sequencing from isolated HSEC

Ulex lectin beads were used to selectively isolate endothelium from tumour (T, purple) and matched non-tumour (NT, green) for five patients. RNA was extracted and sent for deep sequencing. Immune regulatory pathways were downregulated in tumour endothelium, with upregulation of immunomodulatory genes including NT5E (CD73) (arrow). Heatmap and sequencing data supplied by Dr Joanne O'Rourke of the Shetty group.

1.5 CD73 (Ecto-5'-nucleotidase)

1.5.1 CD73 in health

The gene ecto-5'-nucleotidase (NT5E) codes for CD73, a 69kDa ectoenzyme that is found as a GPI-anchored protein and soluble enzyme.[49] [50] It facilitates the production of adenosine in the extracellular space via the CD39/CD73 pathway, whereby CD39 hydrolyses adenosine triphosphate (ATP) to adenosine-diphosphate and -monophosphate (AMP), following which CD73 dephosphorylates AMP to adenosine (Figure 1-7). It is widely expressed in many organs including brain, lung, pancreas, gastro-intestinal tract and reproductive organs.[51] It is also found on the surface of T cells, B cells and afferent lymphatic endothelium.[52] CD73 is present on malignant cells, including lung, colon, bladder, ovarian and papillary thyroid cancer, as well as HCC.[53]

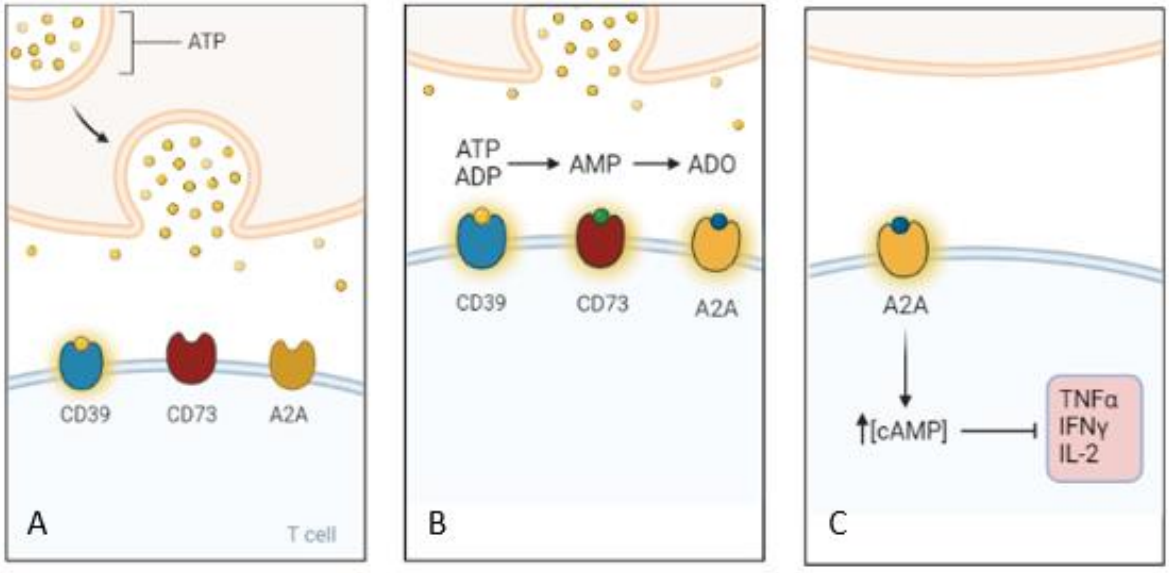


Figure 1-7 The CD39/CD73/adenosine pathway

A) ATP is released from damaged or dying cells and binds to CD39. B) CD39 expressed on the cell surface, in this case on a T cell, converts ATP to AMP in the extracellular space. CD73 then dephosphorylates AMP to adenosine. C) Adenosine binds to one of four subclasses of adenosine receptor. On T cells, binding to A2A receptors leads to increased intracellular AMP, downregulating the release of pro-inflammatory cytokines. ATP = adenosine triphosphate, AMP = adenosine monophosphate, ADO = adenosine, A2A = adenosine receptor, cAMP = cyclic AMP, TNFα = tissue necrosis factor α, IFNγ = interferon γ, IL-2 = interleukin 2. Original figure created using Biorender.

Extracellular adenosine can also be generated via the CD38/CD203a/CD73 pathway using the substrate NAD+ (nicotinamide adenine dinucleotide), for example on the surface of T cells.[54] Adenosine is ubiquitous and has a wide range of functions throughout the body, including within the cardiovascular, central nervous and immune systems. CD73 facilitates the final, irreversible step in adenosine production. Adenosine is difficult to measure in vivo due to its short half-life of 0.6 seconds, and so CD73 can be used as a proxy measure of adenosine and its effects in tumorigenesis, and also represents a potential therapeutic target.[55]

1.5.1 CD73 in Cancer

Adenosine is a nucleotide, a family of signalling molecules that regulate key functions of tissue and are the precursors of nucleosides, necessary for the synthesis of RNA and DNA.[56] Adenosine binds to four G-protein coupled receptors (A1, A2A, A2B and A3) and increases or decreases intracellular cyclic AMP (cAMP) by either inhibiting adenylyl cyclase (A1 and A3) or activating it (A2A and A2B).[57] [58] A2A receptors are expressed on T cells, NK cells and dendritic cells.[59] When adenosine binds to A2A, it leads to increased intracellular cAMP which in turn inhibits the release of pro-inflammatory cytokines such as interferon gamma (IFN-γ), tissue necrosis factor alpha (TNF-α) and interleukin 2 (IL-2), thereby producing an anti-inflammatory effect.[60]

In addition to the effects on immune cell function, CD73 and adenosine also facilitate angiogenesis. CD73 activity is increased under hypoxic conditions via hypoxia-inducible factor 1 alpha (HIF-1α), leading to new vessel formation.[61] This may be mediated by

adenosine binding to A2B receptors, leading to increased intracellular cAMP and VEGF (vascular endothelial growth factor) production on endothelial cells.[62] Allard et al. showed that inhibiting CD73 with a monoclonal antibody reduced VEGF production and tumour angiogenesis in a breast cancer model in mice.[63] Thus, in the hypoxic microenvironment that is characteristic of solid tumours, CD73 may assist cancer growth by promoting angiogenesis and restricting an anti-tumour immune response.[64]

CD73 also has non-enzymatic effects. Overexpression of CD73 promotes proliferation and migration of cervical cancer cells despite blocking its enzymatic function, and is associated with increased production of VEGF, epidermal growth factor receptor (EGFR) and Akt.[65] Therefore, targeting CD73 in cancer therapy may have desirable anti-tumour properties beyond those that modulate immune response.

CD73 is expressed on a range of cancer types. In pancreatic cancer, CD73 expression on tumour cells correlates with perineural invasion and reduced overall survival, and is an independent marker of poor prognosis. Furthermore, incidence of lymph node metastases is significantly associated with CD73 positive tumour infiltrating lymphocytes.[66] Expression is increased on tumour cells in gallbladder cancer compared to healthy tissue, particularly in large, poorly differentiated tumours with metastases.[67] In non-small cell lung cancer, CD73 is expressed on cancer cells, tumour infiltrating lymphocytes and cancer-associated fibroblasts, but not in healthy lung epithelium. Its expression is associated with HIF-1α and lactate dehydrogenase, markers of a hypoxic and acidic tumour environment.[68] Elevated

serum CD73 is associated with poor response to anti-PD-1 immunotherapy and reduced overall survival in melanoma.[69]

Although CD73 in cancer is broadly associated with poorer prognosis, in some malignancies it appears protective. CD73 expression is low in advanced endometrial and ovarian tumours compared to normal tissue, and high CD73 is expressed on less aggressive endometrial cancer, associated with a better prognosis.[70] In acute myeloid leukaemia (AML), CD73 levels were low on malignant blast cells and circulating $CD8^+$ T cells compared to healthy controls. For patients in remission, CD73 expression on $CD8^+$ T cells increased, and the authors suggest that low CD73 expression is implicated in T cell exhaustion and higher expression of the protein is associated with improved anti-tumour response of immune cells in AML.[71] Thus the literature pertaining to CD73 shows that its role in cancer is complex and not uniform across different tumours. This finding is unsurprising given the broad range of functions executed by adenosine. The effect of CD73 in primary liver cancer, however, does appear to be negative, as outlined below.

1.5.2 CD73 in HCC

CD73 is expressed on hepatocytes in normal liver, mainly on the canalicular membrane. It is increased in chronic liver disease, whereby adenosine is implicated in the pathogenesis of steatosis secondary to NAFLD or alcohol [72] [73]. It is also expressed in cancers of the hepatobiliary system, that is, pancreatic ductal adenocarcinoma, cholangiocarcinoma and HCC. Sciarra et al observed a different pattern of CD73 expression in HCC compared to non-tumour tissue, with more intense staining involving the hepatocyte membrane and

cytoplasm in the tumour.[74] CD73 expression in HCC positively correlates with poor differentiation and microvascular invasion, two histological features that represent a more aggressive tumour phenotype and are markers of poor prognosis.[75] CD73 has also been evaluated in association with endothelial-to-mesenchymal transition (EMT), the process by which endothelial cells adopt a phenotype that promotes invasion, immune escape and, ultimately, malignant and metastatic ability. EMT was induced in HCC cells using TNFα, resulting in increased expression of CD73 and the immune checkpoint molecule PD-L1.[76]

Higher expression of CD39 on $FoxP3^+$ regulatory T cells (Tregs) and HCC tumour cells is associated with shorter time to recurrence and reduced overall survival after cancer resection, further demonstrating the significance of the CD39/CD73 adenosine pathway in HCC.[77] Liao et al report increased $CD39^+$ $CD4^+$ T cells in HCC tumour compared to non-tumour tissue, with reduced tumour infiltration of $CD8^+$ T cells. Mice transplanted with Hepa1-6 tumour cells and treated with an A2B adenosine receptor antagonist in combination with sorafenib developed smaller tumours and demonstrated increased $CD8^+$ T cell tumour infiltration compared to monotherapy.[78]

Although increased expression of CD73 has been observed in HCC, Alcedo et al report abnormal glycosylation of CD73 on tumour hepatocytes associated with 3-fold reduction in nucleotidase activity.[79] This suggests that CD73 may promote tumorigenesis via a different mechanism from the adenosine pathway. This theory is further supported by the occurrence of spliced variants. Snider et al report a truncated variant of CD73 that has low expression in normal liver tissue but is upregulated in cirrhosis and HCC. It is 50 amino acids

shorter than canonical CD73 (CD73L), and lacks 5'-nucleotidase activity.[80] CD73L was associated with lower levels of the proliferation marker Ki67 in HCC cells, and was degraded when it formed a complex with CD73S. The authors hypothesise that if CD73L promotes apoptosis, upregulation of CD73S in HCC may lead to reduced CD73L activity and thereby promote cell survival.

Project

CD73 has been implicated in oncogenesis by modulating the immune response, and also via other mechanisms such as angiogenesis. Expression on hepatocytes has been associated with markers of poor prognosis in HCC, but its expression and function in sinusoidal endothelium has not yet been studied.

This project seeks to expand our knowledge of CD73 in HCC, initially performing studies on whole liver samples available to us through established ethics from patients at the pre-cancer stage i.e., those with chronic inflammation of the liver, and in those with established tumours. Following these tissue expression studies, I will then focus on the expression of CD73 on liver endothelial cells. As outlined above, endothelial cells are the key gatekeepers for immune cell infiltration from blood into tissue and therefore are likely to play a critical role in regulating the tumour microenvironment, especially in HCC which is a highly vascularized tumour. Recent positive results from combining immunotherapy with vascular targeting agents provide support that the endothelial cell is a viable target in the tumour microenvironment.[27] Whilst previous studies have been published on CD73 expression and function on liver epithelial cells in both tumour and non-tumour populations, there is limited literature on this receptor's expression and regulation in human liver endothelial cells.

Hypothesis

CD73 expression is increased on hepatocytes and hepatic sinusoidal endothelial cells in hepatocellular carcinoma and contributes to the immunosuppressive tumour microenvironment, leading to negative outcomes in HCC.

Aims

Aim 1: To evaluate CD73 expression in healthy liver tissue and chronic liver disease

Aim 2: To validate RNA sequencing data and study the expression of CD73 in hepatocellular carcinoma and the tumour microenvironment.

Aim 3: To assess how CD73 expression in HCC correlates with prognostic markers and overall survival

Aim 4: To study the expression of CD73 in primary human hepatic sinusoidal endothelial cells and its regulation by factors found in the inflammatory/tumour microenvironment.

Chapter 2 METHODS

2.1 Human Tissue Samples

Archived human tissue was provided by the Queen Elizabeth Hospital Birmingham. Specimens from patients who had undergone liver resection or transplantation were fixed in formalin and embedded in paraffin. Donor livers not used for transplantation was also made available for either normal liver or fatty liver specimens, and were again fixed in paraffin or frozen.

A cohort of 99 HCC cases was selected by a liver pathologist from patients who underwent resection or liver transplantation. Specimens were specifically chosen to include tumour and surrounding tissue to provide an area of matched non-tumour for comparison. Slices were prepared on a microscope slide and fixed in paraffin. Access to all tissue samples was provided by the Human Bioresources Centre (application number 16-270). Clinical data and outcomes up to five years after surgery had been collected for each case and stored pseudonymously.

HSEC were cultured from explant livers or donor livers provided by the Liver Unit, Queen Elizabeth Hospital Birmingham. Ethics were obtained under the title 'Understanding how the immune system promotes the development and progression of tumours in patients with hepatobiliary cancer' (reference number 18/LO/0102).

2.2 Immunohistochemistry

All reagents used are shown in Tables 1 and 2. Paraffin sections were de-waxed and rehydrated three times sequentially in xylene, alcohol and water for two minutes each. After rehydration, sections were incubated in 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity. Sections were then washed in PBS Tween buffer (phosphate buffered saline containing 0.1% Tween-20) three times, for two minutes each. High pH antigen unmasking solution (Tris-based) was prepared by heating 10mls of unmasking solution in 1L of water in a microwave at 850W for five minutes. Slides were then placed in the solution and heated for 15 minutes, then cooled by adding cold water and washed again in PBS-Tween three times for two minutes each. The remainder of the protocol was performed in a humidified chamber.

A border was drawn on the microscope slide around each section using a wax pen. Blocking was performed with a solution of 2x casein in PBS, 250µl/slide and incubated on a rocker for 20 minutes at room temperature (RT). Following this, blocking solution was removed from the slides and the primary antibody added. Anti-CD73 at 0.4µg/ml working concentration diluted in PBS was incubated for 60 minutes on rocker at RT. Slides were then washed in PBS-Tween three times for two minutes each. The secondary antibody was then added to the slides using pre-prepared solution (Vector) and incubated on the rocker for 30 minutes at RT.

The slides were washed again in PBS-Tween three times for two minutes each before adding chromogen Immpact DAB (3, 3'-Diaminobenzidine) for five minutes to aid visualisation. The

slides were washed in tap water for five minutes to remove DAB, then counterstained with filtered Mayer's haematoxylin for one and a half minutes. The slides were dipped briefly in tap water before being washed in water for five minutes. Finally, the slides were dehydrated using water, alcohol and xylene and mounted on cover slips using DPX mounting medium.

A total of 99 HCC sections were stained in batches. DAB was left in situ for ten minutes for the first 50 samples, which created more background staining, therefore, this was reduced to five minutes for the remaining 49 samples. The difference in protocol was adjusted for in the image analysis as discussed below. Normal liver and different chronic disease states were also stained for CD73 expression, and used for isotype matched controls (IMC).

Table 1 Materials for Immunohistochemistry and Immunofluorescence

Material	**Manufacturer**	**Product code**
30% hydrogen peroxide	Sigma-Aldrich	102164296
Methanol	VWR Int	UN1230
Tris-based antigen unmasking solution	Vector	H3301
Casein 10x	Vector	SP-5020-250
DAB Peroxidase substrate kit	Vector Immpact	SK4105
DPX mounting medium	Cell Path	SEA-1304-00A
Goat serum	Abcam	ab138478

Table 2 Antibodies

Primary Antibody	**Origin**	**Manufacturer**	**Product code**	**Working concentration (µg/ml)**
Anti-CD73 (for paraffin)	Rabbit IgG	Atlas	HPA017357	0.4
Anti-CD73 (for HSEC)	Rabbit IgG	Atlas	HPA017357	4
Rabbit IgG polyclonal	Rabbit	Dako	X0903	0.4 (for paraffin) 4 (for HSEC)
Anti-CD31	Mouse IgG1	Abcam	ab9498	5
Mouse IgG1	Mouse	Dako	X0931	5
Secondary antibody				
HRP Anti-rabbit	Horse	Vector	MP-7401	As per manufacturer's instructions
HRP Anti-mouse	Goat	Vector	MP-7452	
Anti-rabbit AlexaFluor 546	Goat	ThermoFisher	A-11035	2
Anti-mouse AlexaFluor 488	Goat	Life Technologies	A21126	2

2.3 Image Analysis

Paraffin sections were individually viewed on Zeiss Vert.A1 microscope. A grading system for the CD73 staining on the HCC cohort was developed with a consultant histopathologist. This qualitatively assessed the distribution of staining of hepatocytes as canalicular, membranous or cytoplasmic, and noted whether there was positive staining of vascular endothelia. I graded the intensity of sinusoidal staining in the tumour relative to the adjacent non-tumour tissue as follows: 0 = no positive staining in tumour, 1 = less than non-tumour, 2 = the same, 3 = greater than non-tumour. The grading was performed blind to any clinical information about the case.

The HCC cohort was also imaged using a Zeiss AxioScan Slide Scanner. Five fields of view at 2.5x magnification were taken at random for non-tumour and tumour using ZEN imaging software. Each field of view was then analysed using ImageJ software (Reuden) to quantify the intensity of staining as a percentage. IMC samples were used as a comparator to select a user-defined threshold for positive staining. The threshold was set at 180 for the first cohort of 37 cases, and 170 for the second cohort of 37 cases, as there was a difference in staining intensity with duration of DAB incubation (five minutes or ten minutes). The mean was calculated from the five results for tumour and non-tumour respectively. 25 samples did not have exact alignment of the coverslip with the microscope slide and therefore they could not be imaged using the Slide Scanner. However, all 99 samples were assessed qualitatively.

2.4 Immunofluorescence

Primary human HSEC were provided by the Shetty group, and were isolated by immunomagnetic selection for CD31 surface expression from mechanically minced normal or diseased liver tissue as described by the manufacturer (ThermoFisher). After being cultured for 24 hours, HSEC were treated with IFN-γ and TNF-α at 10ng/ml for a further 24 hours, or left untreated. Confluent monolayers of HSEC were then fixed in 4% paraformaldehyde (PFA) in a six channel µ slide (Ibidi). PFA was removed and the cells washed three times with 100µL PBS-Tween, then placed on a rocker for five minutes at RT. HSEC were then washed three times with a blocking buffer consisting of PBS, 10% goat serum and 2x casein. The third wash was kept in situ for incubation for 30 minutes on a rocker. The blocking buffer was then removed and primary antibody added (rabbit anti-CD73, Atlas, at 4µg/ml working concentration in PBS or rabbit polyclonal IMC at an

equivalent concentration, and mouse anti-CD31, Abcam, at 5µg/ml or mouse IgG1 IMC) and incubated for 60 minutes on a rocker at RT. The primary antibody was then removed from the µ slide and each channel washed with PBS-Tween as described above. The fluorescently-labelled secondary antibody was washed through three times. Following the third wash the solution was left in situ for incubation on a rocker for 30 minutes at RT, with the µ slide wrapped in foil to protect it from light. The secondary antibody was then removed, and the cells washed with PBS-Tween twice as described above. Following the third wash the solution was left in situ, the µ slide wrapped in foil and left on rocker for five minutes at RT. Two further washes were performed with PBS-Tween solution. The cells were then incubated with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride at 300nM concentration) for nuclear staining, and incubated in foil for two minutes. The DAPI was then removed, the cells washed in PBS-Tween three times, with the final wash left in situ. The µ slide was wrapped in foil and stored at 4°C. HSEC were imaged using Zeiss LSM780 Confocal microscope.

2.5 Quantitative RT-PCR

cDNA generated from human liver cubes, HSEC and the cell lines Huh7 and HepG2 was provided by the Shetty group. The immortalised cell lines were originally created from HCC tumour cells. Isolated HSEC were submitted to shear stress by placing them on an orbital shaker at 1Pa for 72 hours before RNA extraction. The materials used for qPCR are summarised in Table 3. The experiment was conducted on ice. Pipettes, tips and Eppendorfs were first treated with UV radiation for 20 minutes to destroy any nucleic acid contaminants. Master mixes were created for each primer, consisting of 5.00µL 2xTaqMan

stock mastermix, 4.25µL nuclease free water and 0.25µL primer probes per reaction. The TaqMan probe contains a 5'fluorescent dye and a 3'quencher. Master mix was pipetted into a 384 well plate. For each reaction, 0.5µL of cDNA was added to give a total volume of 10µL per well. Three CD73 primer-probe sets were used, one for the canonical gene, one for the spliced variant, and one that should recognise both forms of the CD73 transcript. Nuclease free water was used in place of cDNA for a negative control. GAPDH housekeeping gene was used for HSEC, Huh7 and HepG2. 18S housekeeping gene was used for whole liver. All primers are manufactured by TaqMan. The primer contains a proprietary probe; the full primer sequences are not available.

Once the plate had been loaded it was sealed with a clear plastic cover, and placed in a centrifuge for three minutes at 200rpm. The plate was then placed in a LightCycler480 for the PCR run. PCR data were analysed using Microsoft Excel and GraphPad Prism.

Table 3 Materials for qPCR

Material	**Manufacturer**	**Product code**
TaqMan Gene Expression Master Mix	Applied Biosystems	4369016
Nuclease free water	Promega	P119C
Primer probes		
CD73 L	ThermoFisher	Hs01573922
CD73 S	ThermoFisher	Hs04234687
CD73 combined	ThermoFisher	Hs00159686
GAPDH	ThermoFisher	Hs99999905
18S	ThermoFisher	Hs99999901

2.6 Statistical Analysis

Analysis of results was performed using Microsoft Excel and GraphPad Prism 9 software. Kaplan-Meier curves were analysed using log rank Mantel-Cox test. Non parametric data were analysed using Fishers exact test or Chi square test. Paired data were analysed using paired t-test. P values <0.05 were deemed statistically significant.

Chapter 3 THE HEPATIC EXPRESSION OF CD73 IN HEALTH AND CHRONIC DISEASE

3.1 Introduction

As discussed in Chapter 1, the nucleotidase CD73 is expressed in normal liver and in primary liver cancer, hepatocellular carcinoma (HCC). However, there is little reported in the literature regarding the expression of CD73 in health and different chronic liver diseases. In order to better understand the role of CD73 in HCC, I first sought to establish where CD73 is expressed in normal liver at the protein level. I performed immunohistochemistry (IHC) on sections of normal liver and chronic liver disease.

All the experiments presented have been performed using human liver tissue supplied by the Liver Unit at the Queen Elizabeth Hospital, Birmingham. By collaborating with one of the largest liver transplant centres in the world we have access to diseased livers from patients undergoing liver transplantation due to a wide range of chronic liver conditions. HCC tumours are provided following surgical resection or transplant. Normal liver tissue is also made available from donor organs that are unsuitable for transplantation, for example due to prolonged ischaemic time or steatosis. Although these livers may not therefore be considered 'healthy', they are all non-cirrhotic without evidence of chronic disease or fibrosis. IHC slides were reviewed by Dr Owen Cain, a consultant histopathologist who specialises in liver disease. He gave expert opinion on the pattern of staining and interpretation of which area of tissue or cells were positive.

Aim 1: To evaluate CD73 expression in healthy liver tissue and chronic liver disease

3.2 Localisation of CD73 in normal liver tissue

Immunohistochemistry (IHC) was performed on paraffin sections of normal liver (NL) tissue. A working concentration of anti-CD73 antibody at 0.4µg/ml was recommended by the manufacturer (Atlas) which produced crisp staining and therefore this concentration was used for all IHC. In NL there was positive staining on sinusoids and canaliculi, representing CD73 expression on endothelial and epithelial cells respectively. For context, Figure 3-1 shows the anatomy of a liver lobule and portal triad in human tissue, as previously depicted as a schematic in Figure 1-4 of Chapter 1.

Table 4 Patient demographics of normal liver samples

CLR number	Gender	Age (years)	Ethnicity	BMI	Type of donor
3106	M	71	White	32	DCD
3126	F	61	White	28	DBD
3129	M	52	White	28	DBD
3332	M	77	White	22	DBD
4120	M	66	White	26	DBD

CLR (Centre for Liver Research) identifying number; Gender M = male, F = female; BMI (Body Mass Index); Type of donor, donor after cardiac death (DCD), donor after brainstem death (DBD)

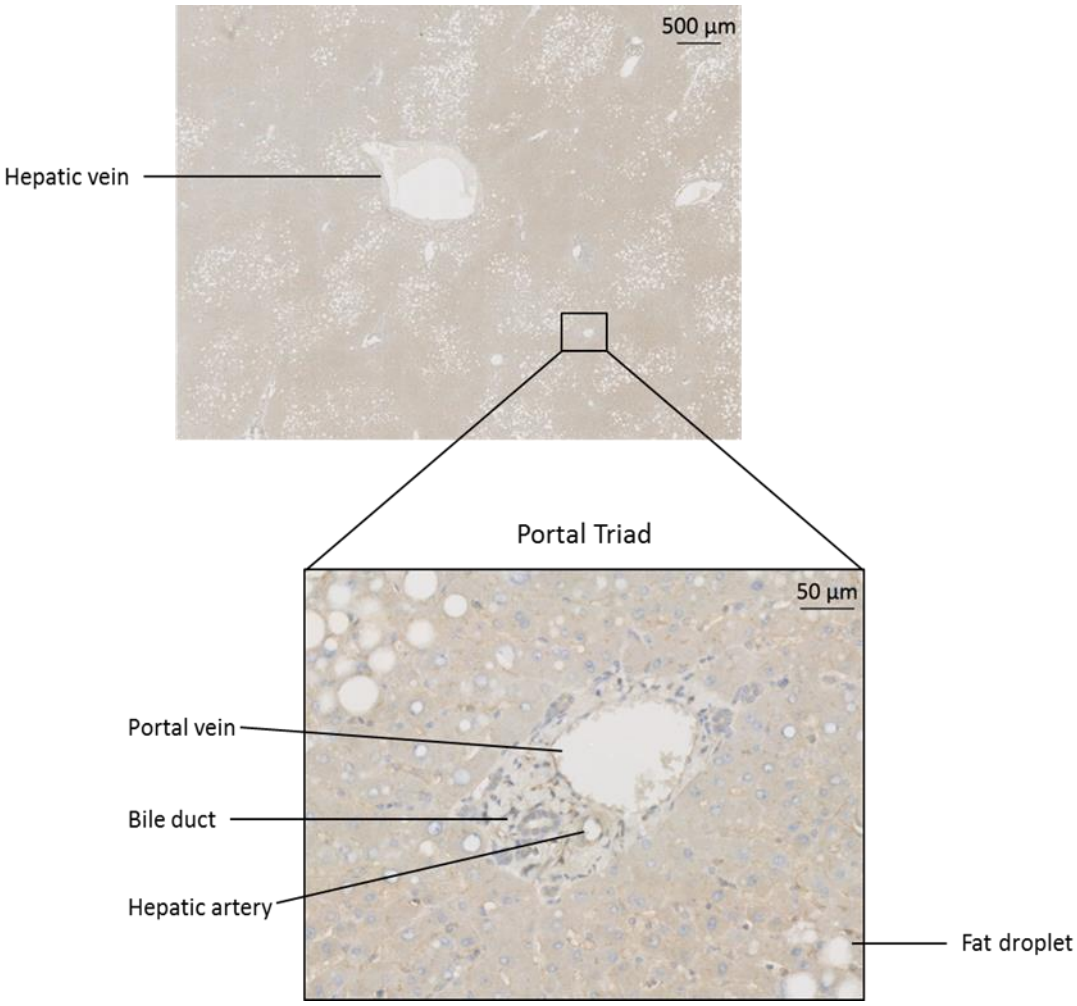


Figure 3-1 Example of a liver lobule and portal tract in normal liver tissue

Paraffin embedded section of normal liver tissue stained with anti-CD73 (brown). A = low power view showing a liver lobule. A central vein is surrounded by portal triads, highlighted at 10x magnification in image B. A bile duct and branch of the hepatic artery are in close approximation to a branch of the portal vein to form a portal tract, or triad. Blood from the portal vein and artery combines to flow through sinusoids to the hepatic vein. Bile flows in the opposite direction, from the centre of the lobule towards the portal tracts, where it drains into ducts. This sample has a high fat content (steatosis) due to the presence of fat droplets, as indicated.

3.3 Normal liver sinusoids and canaliculi express CD73

IHC was performed on paraffin sections of normal liver tissue as described in Chapter 2. This showed positive staining of sinusoids in all specimens. Positive staining for canaliculi, 1µm diameter channels formed between adjacent hepatocytes was also observed.

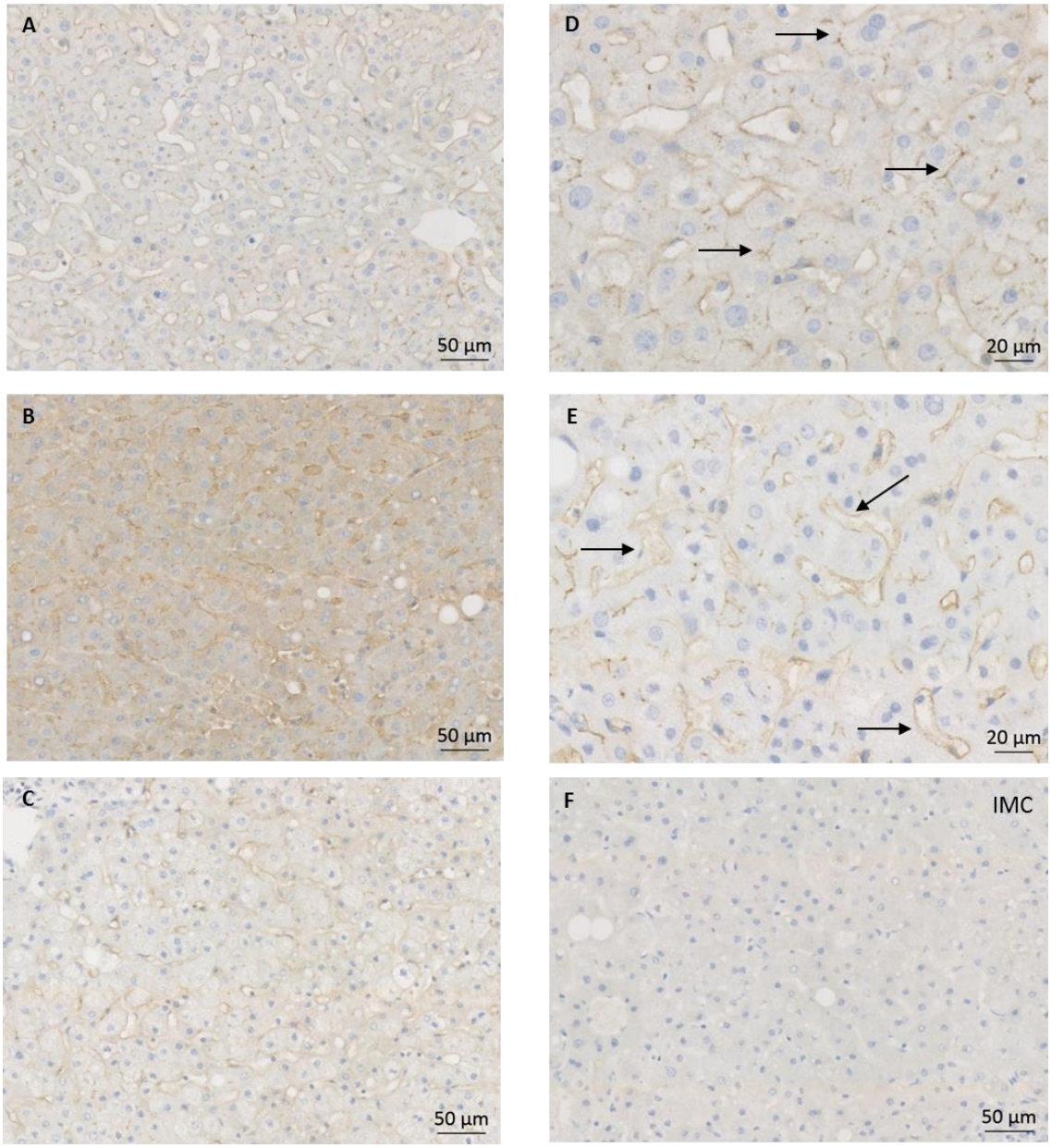


Figure 3-2 Sinusoids and canaliculi express CD73 in normal liver

A-C) Low power magnification of NL, each image is taken from a different tissue sample. IHC for CD73 (brown) shows staining of canaliculi and sinusoids. High power images on further cases show D) positive staining of canaliculi (arrows) and E) sinusoids (arrows). F) Isotype matched control (IMC) indicating low levels of non-specific binding to the tissue.

3.3 CD73 is expressed in a range of chronic liver diseases

Although the causes of chronic liver disease are broad, including viruses, genetic mutations and autoimmune disease, persistent inflammation of any aetiology will ultimately lead to

fibrosis and permanent scarring of the liver parenchyma, termed cirrhosis. This is associated with significant architectural change as highlighted in Figure 3-3. The expression of CD73 has been studied in mouse models of alcohol induced liver disease, but descriptions of CD73 staining in cirrhosis in humans are limited to small numbers in ArLD and viral hepatitis only.[74] [81] [74, 82] I performed IHC on human samples of alcohol related liver disease (ArLD) and non-alcoholic steatohepatitis (NASH), together with two autoimmune conditions, primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH), to gain an insight into the regulation of CD73 expression across the spectrum of chronic liver disease (Table 5).

Table 5 Patient demographics of chronic liver disease tissue samples

CLR number	Gender	Age (years)	Ethnicity	Disease	UKELD score	Alb (g/L)	Bili (µmol/L)	INR	Plt ($x10^9$/L)
4779	F	41	White	AIH	59	26	180	1.7	91
4192	M	19	White	AIH	56	31	97	2.2	50
4293	F	50	White	AIH	48	35	51	1.1	160
4807	F	19	Asian/Indian	AIH	62	27	149	1.6	109
5571	F	70	White	AIH	n/a	22	82	1.8	172
4826	F	67	White	PBC	n/a	29	18	1.6	71
4874	M	58	n/a	PBC	n/a	38	64	1.3	179
5953	F	29	White	PBC	53	15	92	1.2	206
5006	F	64	White	PBC	n/a	32	70	1.4	86
5518	M	65	White	PBC	50	21	53	1.4	73
2038	F	66	White	ArLD	n/a	28	23	2	40
2336	M	57	White	ArLD	54	27	32	1.7	131
3105	M	47	n/a	ArLD	n/a	35	108	2.4	72
3566	M	52	n/a	ArLD	53	27	67	1.5	31
3892	M	51	White	ArLD	54	33	64	1.6	103
4127	F	62	White	NASH	n/a	38	39	1.6	66
3372	M	56	White	NASH	n/a	22	45	1.7	111
3518	M	46	White	NASH	51	26	50	1.4	25
2769	M	31	White	NASH	n/a	31	89	1.7	54
3610	M	64	White	NASH	56	23	1142	2	49

CLR (Centre for Liver Research) reference number; Gender M = male, F = female; Disease AIH (autoimmune hepatitis), PBC (primary biliary cirrhosis), ArLD (alcohol related liver disease), NASH (non-alcoholic steatohepatitis), UKELD (United Kingdom model for End-stage Liver Disease), Alb (albumin), Bili (bilirubin), INR (International Normalised Ratio), Plt (platelets); n/a (not available).

As seen in normal liver, positive staining for CD73 was observed in sinusoids and canaliculi and was similar across the range of diseases. There appeared to be more intense staining of canaliculi in PBC, a biliary disease. Samples of AIH that had a high inflammatory cell infiltrate, as determined by histological appearance, also appeared to have stronger staining, although this is a qualitative observation and not a quantitative assessment (Figure 3-4).

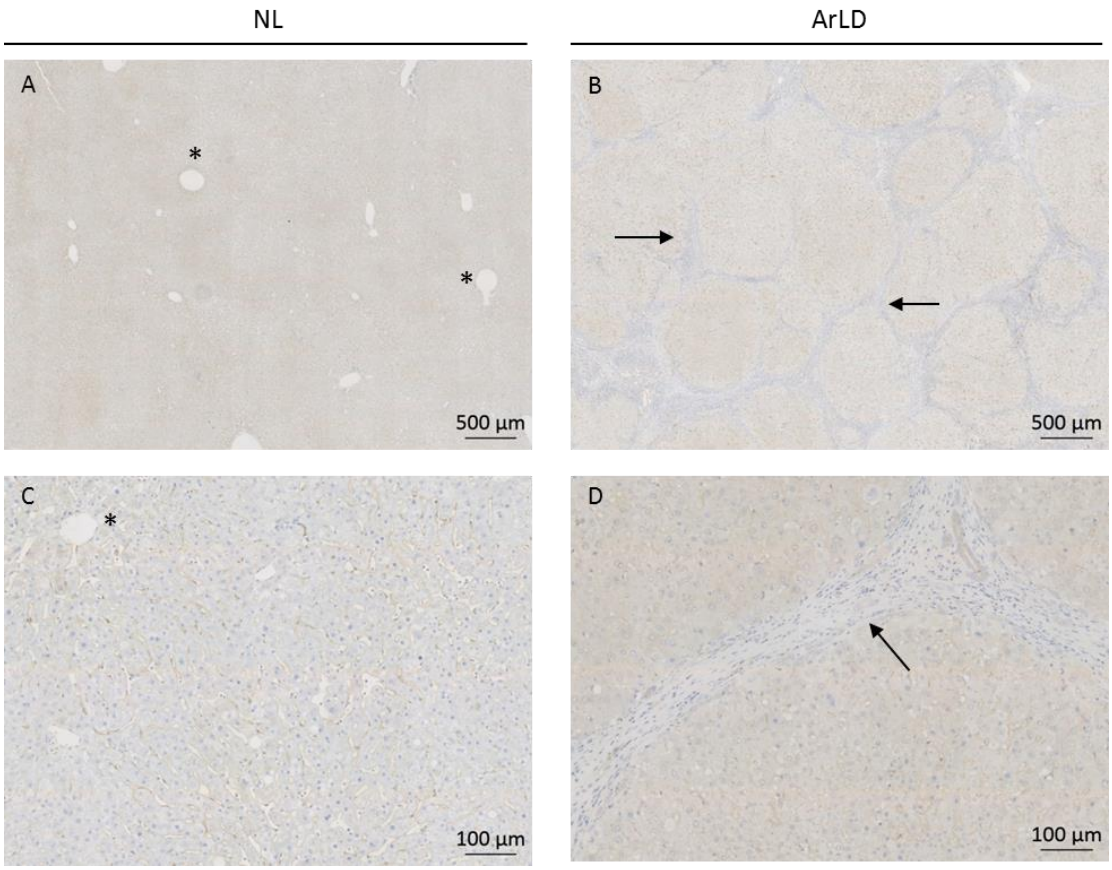


Figure 3-3 Development of nodules and fibrous bands in liver cirrhosis

A) Low power view of normal liver (NL) demonstrating uniform morphology of the parenchyma. Central veins in the middle of liver lobules are visible (asterisk). B) Alcohol related liver disease (ArLD) where the liver has become scarred due to chronic injury. Cirrhotic nodules are clearly demonstrated, surrounded by fibrotic bands of tissue (arrows) containing vessels and a dense inflammatory cell infiltrate (blue), producing a cobblestone appearance. Between fibrous bands are islands of hepatocytes that have lost the central vein. CD73 (brown) is present in sinusoids, canaliculi and endothelium of larger vessels in NL and ArLD, but not the scar tissue. C) High power view of NL with central vein (asterisk). D) High power view of ArLD with fibrous band (arrow).

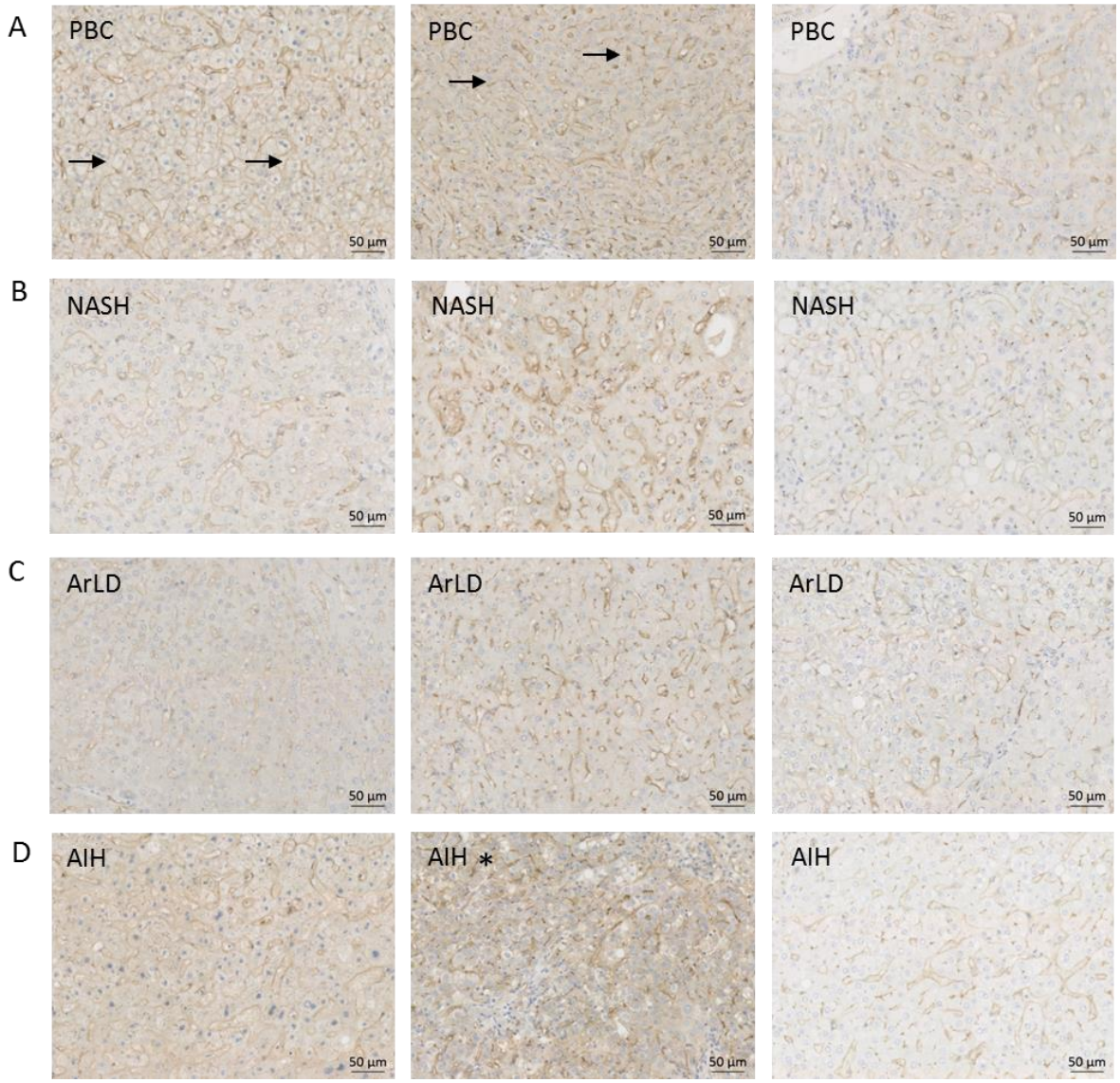


Figure 3-4 CD73 is expressed on sinusoidal endothelium and canaliculi in chronic liver disease

As seen in health, CD73 is expressed on sinusoids and canaliculi in a range of chronic liver diseases (A-D). Staining intensity is similar across a range of conditions. Prominent staining of CD73 is noted on canaliculi in the biliary disease PBC (arrows), and a highly inflamed sample of AIH (asterisk). PBC = primary biliary cholangitis, NASH = non-alcoholic steatohepatitis, ArLD = alcohol related liver disease, AIH = autoimmune hepatitis. Each field of view is taken from a different sample, n=12.

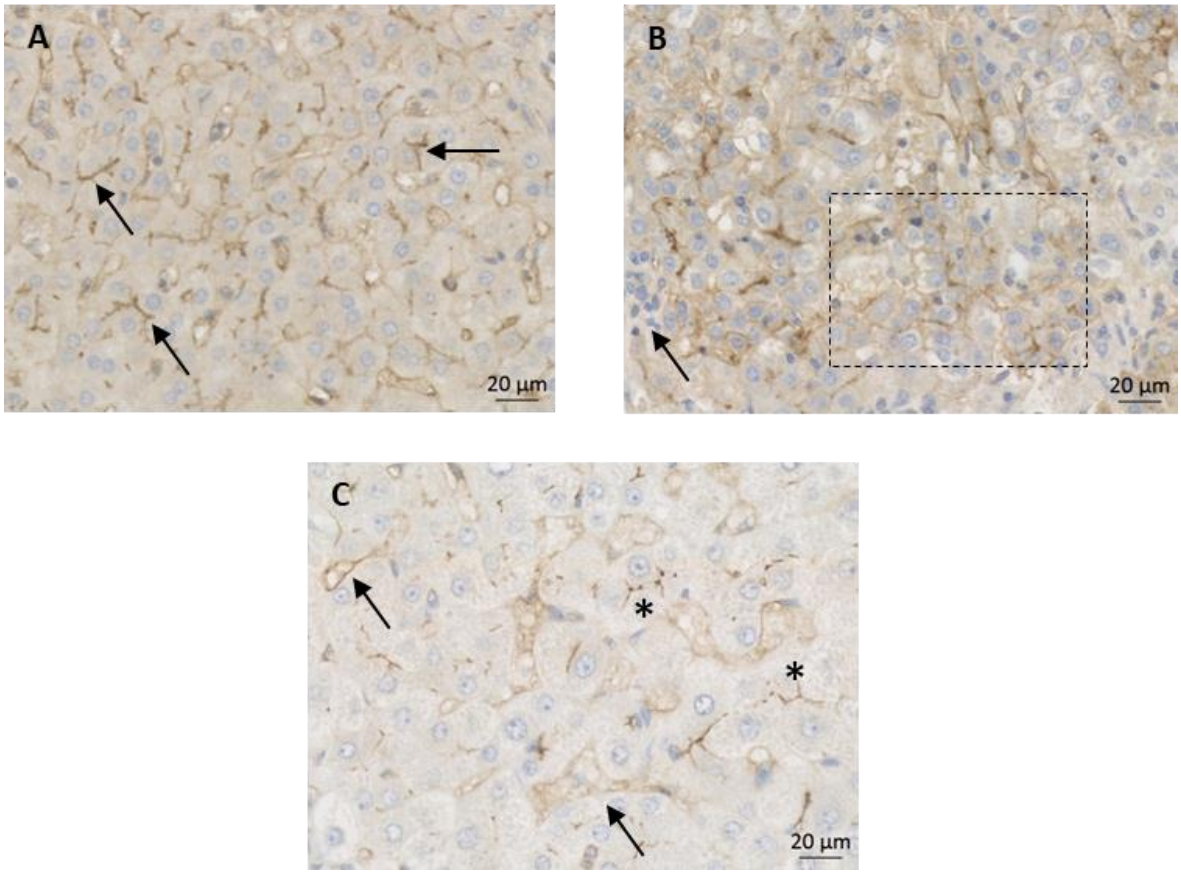


Figure 3-5 CD73 staining of canaliculi in PBC and hepatocytes in AIH is stronger compared to other CLD

A) High power view of PBC, with intense staining in canaliculi (arrows). B) AIH with inflammatory cell infiltrate (arrow) and stronger staining around hepatocytes which are densely packed together (box) when compared with C) ArLD, showing CD73 staining in sinusoids (arrow) and canaliculi (asterisk).

3.4 Discussion

Chronic liver disease is initiated by an insult to hepatocytes, such as alcohol or hepatitis C. Damaged and apoptotic hepatocytes activate immune cells by releasing DAMPs and pro-inflammatory cytokines such as CCL2, TNF-α and IL-6.[83] Quiescent hepatic stellate cells become activated and, with other myofibroblasts, lay down additional extracellular matrix (ECM) within the space of Disse. The ECM distorts sinusoids and their fenestrated endothelium, causing loss of permeability between sinusoid channels and hepatocytes.[84] As a result, blood from portal tracts is directly shunted into central veins and hepatocyte

function is impaired. Over time this leads to development of cirrhotic nodules surrounded by fibrous bands and hepatic vascular resistance increases.[85] These changes may manifest clinically as jaundice, coagulopathy and portal hypertension, and are also precursors to the development of HCC. Although the key steps in the pathogenesis of cirrhosis are well described, the mechanisms that mediate this process are still poorly understood.

I have shown that CD73 is expressed on canaliculi and sinusoids in normal liver tissue and chronic disease, i.e. on epithelial and endothelial cells which are both key elements involved in the development of fibrosis. Bile is produced by hepatocytes and actively transported into canaliculi, which drain into bile ductules, then bile ducts and ultimately the hepatic duct, which empties into the gallbladder.[86] Here bile is stored until required post-prandially to facilitate fat absorption in the small intestine. Therefore, canaliculi may be considered the capillaries of the biliary system. The IHC findings are consistent with published data, but limited by their descriptive nature, as it is not possible to determine whether stronger staining observed on canaliculi in chronic biliary disease (PBC) and in highly inflamed tissue in AIH represents a bystander effect, or a functional contribution of CD73 to the pathological process.[74] However, previous studies have shown dynamic changes in the ATP/CD73 axis in the diseased state. ATP is a danger signal of cell injury, and is released by damaged hepatocytes.[87] Hepatic stellate cells have also been shown to increase expression of CD73 upon activation.[88] Therefore, cell surface expression of CD73 could increase in response to inflammation, as seen in the qualitatively stronger staining on canaliculi in biliary disease. Sinusoids are positive for CD73 in NL and chronic disease, suggesting that adenosine production has a role in physiological and pathological states. It would be interesting to

stain liver sections for CD73 and markers of immune cells such as CD68 for macrophages or CD3 and CD4 for T cells, to further investigate whether there is a relationship between CD73 and sites of inflammation, CD73 has been described on immune cells such as Tregs, NK cells and CD4+ lymphocytes in inflammatory disease and malignancy.[89] I did not detect any positive immune cells in this cohort, which may be due to the low numbers, or absence, of lymphocytes in these samples. Patterns of expression could also be established by co-localising CD73 with markers of endothelium (CD31) and hepatocyte canalicular membrane (occludin). The limitations associated with the qualitative data analysis could be resolved by increasing the sample size for IHC in CLD and quantifying the area of positive staining using ImageJ, as described in Chapter 2, although this is not the focus of this project. However, the results obtained are interesting as they show a variation in spatial distribution of CD73 in cirrhosis, and I am not aware of previous reports in the literature describing CD73 across this range of chronic liver diseases.

After demonstrating the expression of CD73 in normal liver and CLD, I wanted to explore its expression in HCC, as the preliminary sequencing data suggested that the tumour environment would be rich in CD73 expression. I therefore planned to evaluate whether expression of CD73 differs in the tumour microenvironment compared to liver cirrhosis.

Chapter 4 EXPRESSION OF CD73 IN HCC

4.1 Introduction

After establishing the protein expression pattern of CD73 in normal liver and chronic liver disease tissue sections, I next wanted to characterise and compare its expression in HCC. As discussed in Chapter 1, increased CD73 has been reported in HCC and is associated with markers of poor prognosis. I sought to corroborate these findings, with a view to addressing the following aims:

Aim 2: To validate RNA sequencing data and study the expression of CD73 in hepatocellular carcinoma and the tumour microenvironment.

Aim 3: To assess how CD73 expression in HCC correlates with prognostic markers and overall survival

4.2 CD73 is expressed on the hepatocyte membrane and cytoplasm in HCC

I performed IHC on 99 different patient samples of HCC tumours. With one exception, each paraffin embedded section included some surrounding background liver tissue to enable matched comparison of staining between tumour and non-tumour. The pattern of IHC staining in HCC tumours was different from NL and CLD. In HCC I observed positive staining for CD73 around the hepatocyte membrane, or as a stippled effect within hepatocyte cytoplasm (Figure 4-1). Membranous staining in the tumour was observed in 40 cases out of 99. Only two cases had membranous staining in non-tumour, therefore membranous staining has a specificity of 97% for malignant hepatocytes in this cohort. Furthermore, cytoplasmic staining was noted in 14 cases and was unique to tumour tissue.

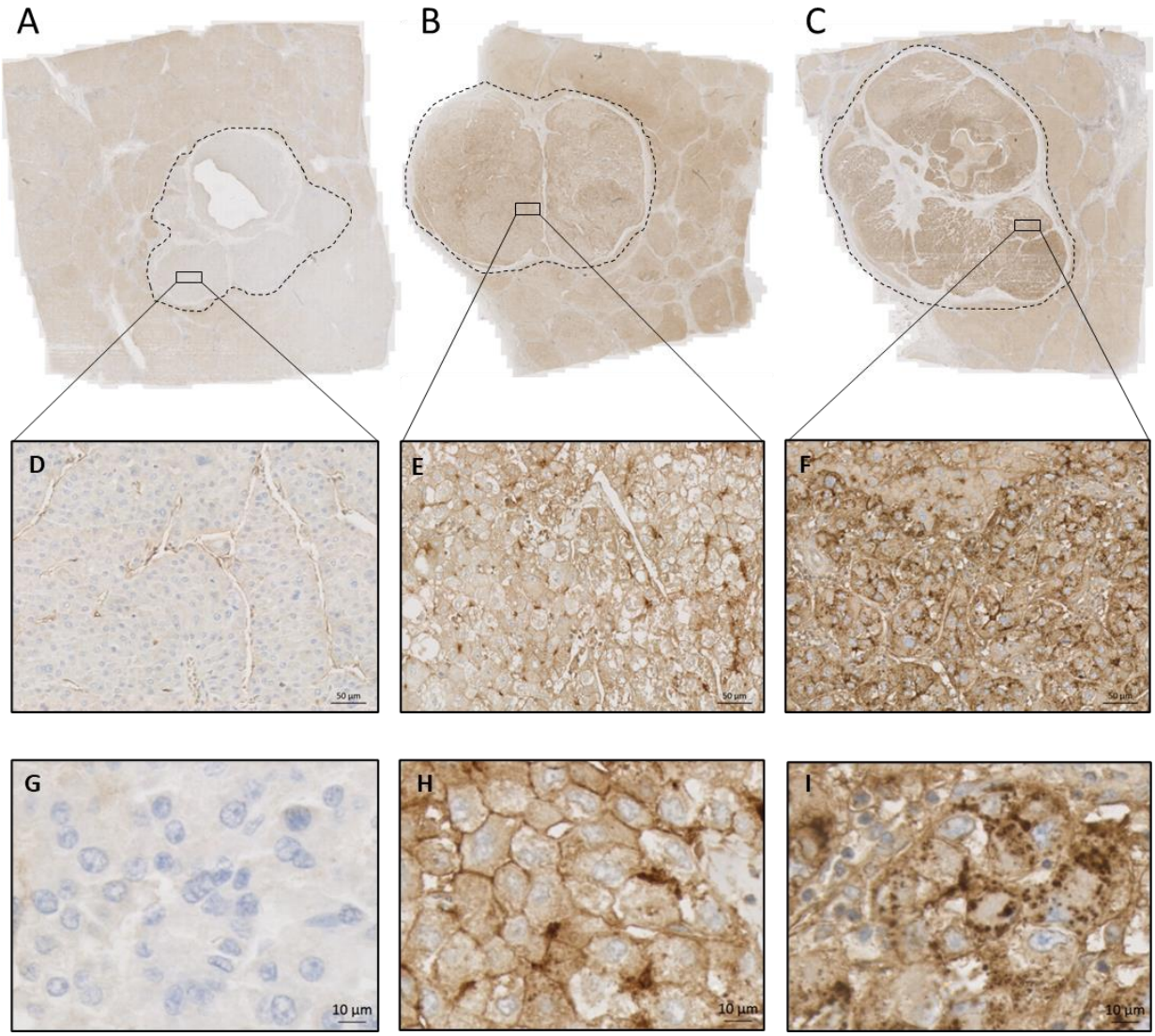
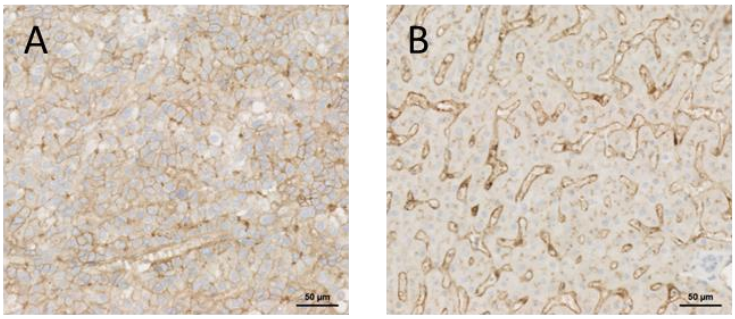
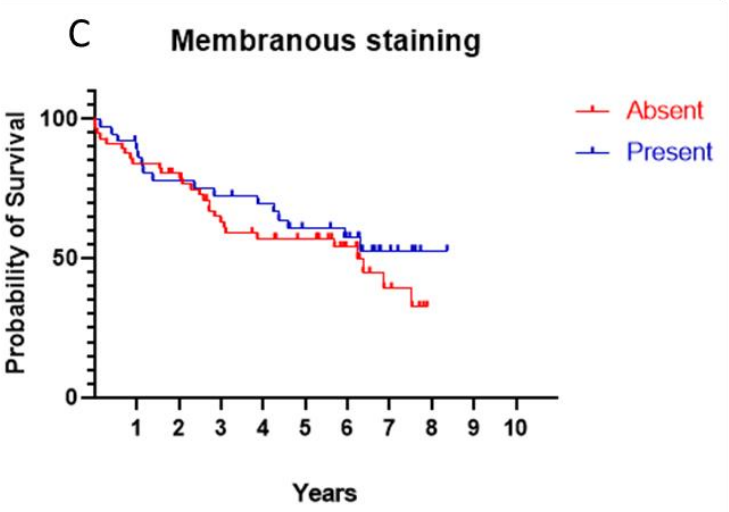


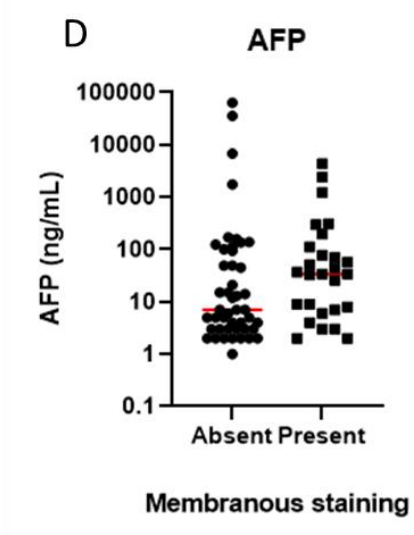
Figure 4-1 CD73 expression in HCC tumours is variable and includes staining of hepatocyte membrane and cytoplasm.

Whilst staining for CD73 in non-tumour tissue was uniform and matched the results from IHC in normal liver and cirrhosis, I found a wide range of patterns of CD73 expression in hepatocellular tumours. Examples of variation shown by: A) Sinusoidal staining only, which is less prominent in the tumour compared to non-tumour tissue. The central area of the tumour is necrotic. B) Membranous staining of hepatocytes in the tumour, CD73 positivity is similar between tumour and non-tumour. Cirrhotic nodules are evident in the non-tumour tissue. C) Dense staining of hepatocytes involving both the cytoplasm and cell membrane produces more prominent staining of CD73 in the tumour compared to surrounding non-tumour. D-F) Medium power views of tumour sections (scale bar 50µm). G) High power view of minimal sinusoidal staining, hepatocytes are bland. H) Membranous staining of hepatocytes produces a 'chicken wire' appearance. I) Cytoplasmic staining creates a granular appearance in hepatocytes. Tumour capsules are highlighted by dashed lines.

I then compared membranous staining with clinical data, looking at overall survival and factors associated with poor prognosis: stage of tumour differentiation, vascular invasion and the serum tumour marker alpha fetoprotein (AFP). Tumour differentiation is a histological assessment of how similar the tumour morphology is to the tissue of origin. Tumours were graded by a pathologist blind to the CD73 results as well, moderate or poorly differentiated. Poorly differentiated tumours are histologically very different from the original cell type, and generally carry a worse prognosis than well differentiated tumours. Although membranous expression of CD73 was tumour specific, there was no significant association with overall survival, stage of tumour differentiation or AFP. Membranous staining was associated with a higher incidence of vascular invasion, but this did not reach statistical significance (Figure 4-2). The full grading of IHC for analysis is shown in Table 7 at the end of the chapter. Five cases were excluded from analysis due to absence of clinical data.







E

Membranous staining	Vascular invasion	No vascular invasion	Total
Present	27	10	37
Absent	31	25	56
Total	58	35	93

F

Membranous staining	Well	Moderate	Poor	Total
Present	6	26	5	37
Absent	14	34	6	54
Total	20	60	11	91

Figure 4-2 Membranous staining of CD73 in HCC is not associated with survival, vascular invasion, tumour grade or serum AFP levels.

Examples of A) membranous staining and B) non-membranous staining in HCC tumours. C) Membranous CD73 expression was not associated with a difference in overall survival (n = 99, p = 0.376 log-rank Mantel-Cox test), D) serum AFP, E) vascular invasion (n = 93, no clinical data for 6 samples, p = 0.126 Fishers exact test) or F) stage of differentiation (n = 91, no clinical data for 8 samples, p = 0.542 Chi square test).

4.3 Sinusoidal staining of CD73 is variable in HCC

As there was variation in staining intensity for CD73 between tumour and non-tumour, I wanted to measure the difference and assess whether it correlated with clinical outcomes. Semi-quantitative analysis was performed by comparing sinusoidal expression of CD73 in the tumour with matched non-tumour from the same specimen. A sample of the grading was corroborated by a liver histopathologist. Seven cases were excluded, one as there was no non-tumour tissue for comparison, and another where the tumour had grown at the edge of the liver and had compressed surrounding non-tumour tissue against the liver capsule, giving a false appearance of intense staining where the sinusoids were compacted together. As before, five cases did not have any clinical information available. The majority of samples had lower or equal expression of CD73 in the tumour compared to non-tumour (table 4). There was no difference in overall survival or grade of differentiation according to sinusoidal grade of staining (Figure 4-3).

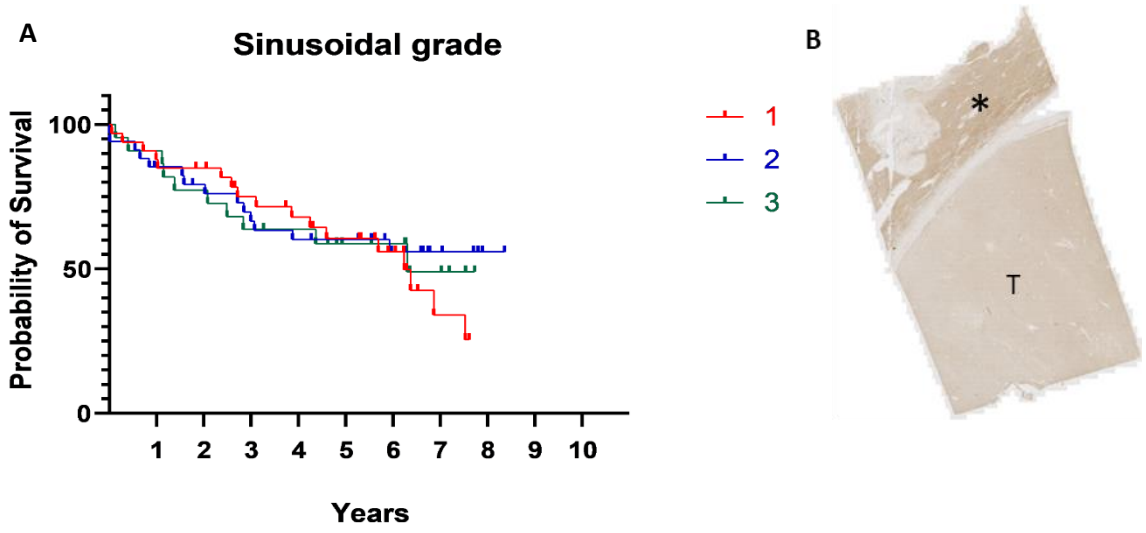


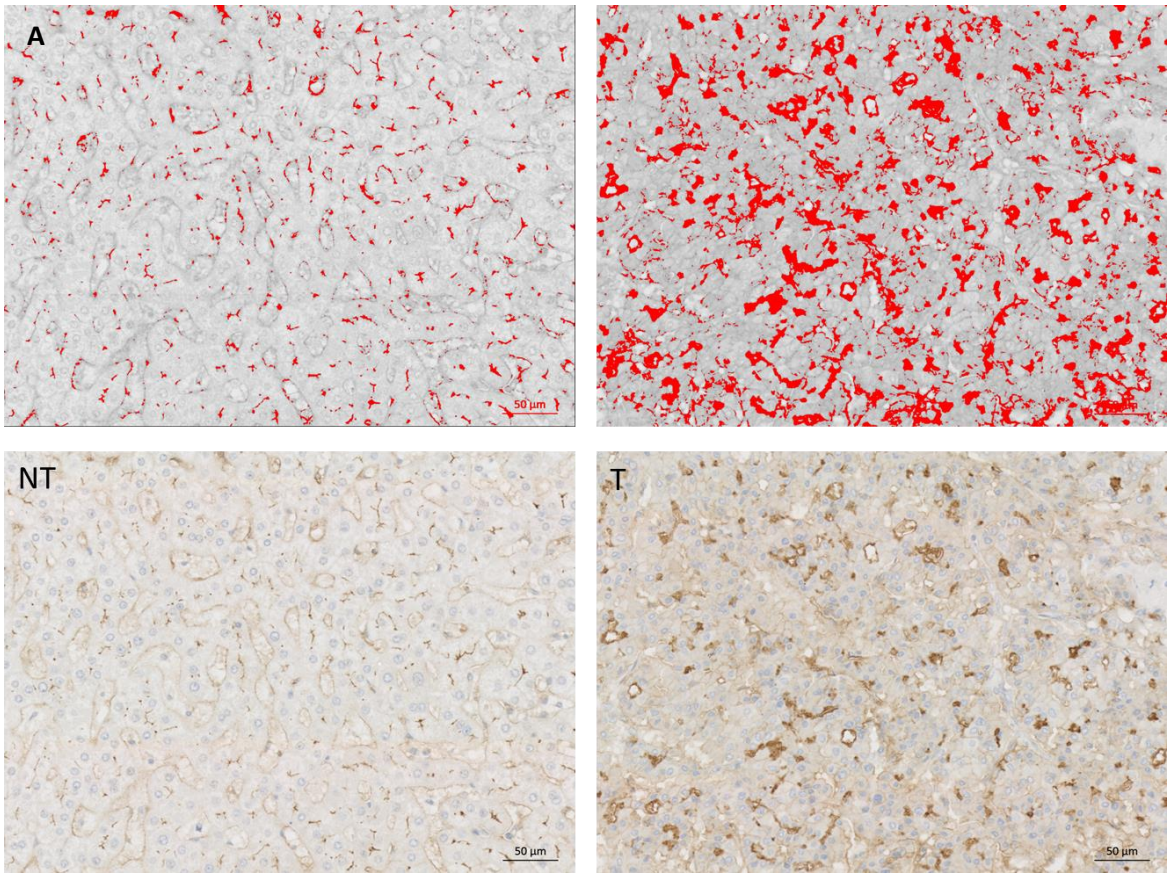
Figure 4-3 Different grades of sinusoidal staining of CD73 in HCC are not associated with a change in survival

A) Different grades of sinusoidal staining for CD73 did not show a difference in overall survival (n = 92, p = 0.836 log-rank Mantel Cox test). B) Tumour sample excluded from analysis due to proximity to liver capsule (asterisk).

Table 6 Semi-quantitative analysis of sinusoidal expression of CD73

Grade	Expression of CD73 on sinusoids in tumour relative to non-tumour	n of cases
0	Absent	3
1	Lower	33
2	Equal	34
3	Greater	22

Further analysis of CD73 staining was performed quantitatively by measuring the intensity of staining using ImageJ on 74 cases. Five areas were selected at random from tumour and non-tumour in each sample, and positive staining was measured as a percentage of the total observed area. Mean average of CD73 positive staining was calculated for tumour and non-tumour for each case. A positive difference between tumour and non-tumour was defined if the mean percentage for each section varied by 10% or more. Cases where the staining areas for tumour and non-tumour were within 10% of each other were defined as having no difference. Based on this criterion, there was a trend of improved overall survival in the CD73 low group compared to equal or high, although this did not reach statistical significance ($p = 0.70$). Interestingly, the worst survival was seen in the 'CD73 equal' group (Figure 4-4).



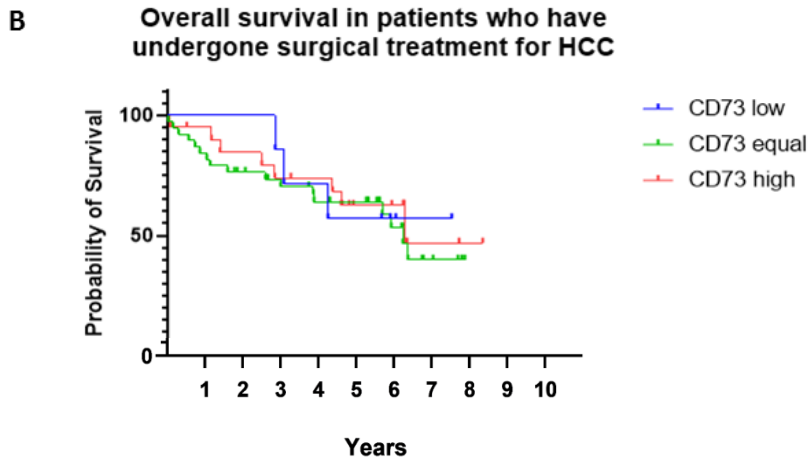


Figure 4-4 there is no difference in overall survival by quantitative analysis of CD73 expression

A) Representative images of one field of view from non-tumour (NT) and tumour (T) with corresponding threshold analysis using Image J (red images) measuring proportion of total area showing positive staining for CD73 of 2% and 15% respectively. B) Quantification of CD73 positive staining was not associated with change in overall survival (n=74, p = 0.70 log rank Mantel Cox test).

4.4 CD73 is expressed on peri-tumour vessels

HCC tumours often develop a tumour capsule - a surrounding band of fibrous tissue containing vessels and inflammatory cells. Whilst the tumours had a variable CD73 intensity and expression pattern, IHC on all samples from the HCC cohort demonstrated positive staining for CD73 on the endothelium lining peri-tumour vessels, bar one case that was negative throughout. I could not detect any obvious positive staining on inflammatory cells in any of the cases. Peri-tumour vasculature could contribute to the increased expression of CD73 in tumour endothelium that was shown by the RNAseq data, and thus suggests that the tumour capsule could play a contributory role to the immunosuppressive tumour microenvironment (Figure 4-5).

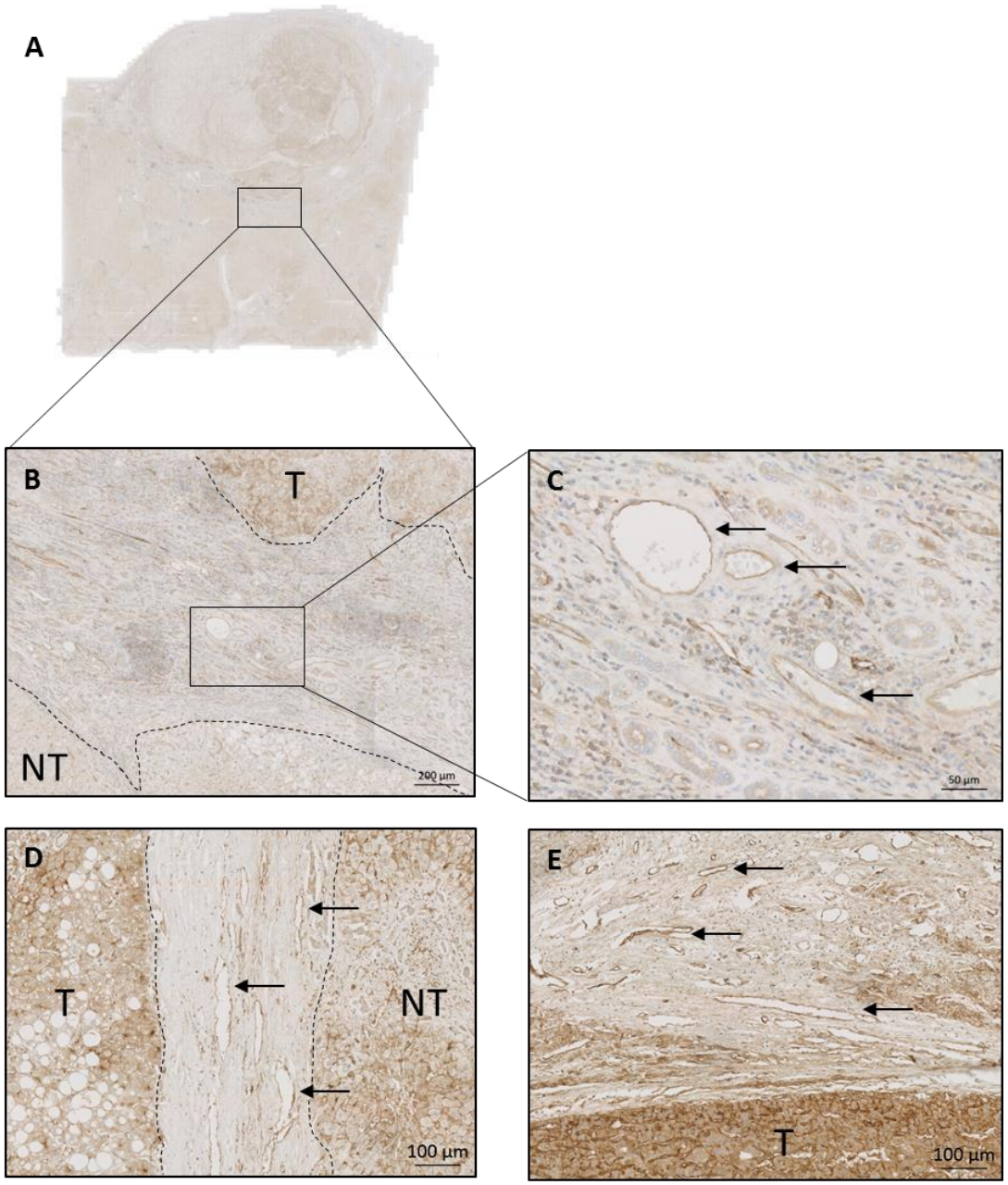


Figure 4-5 CD73 is expressed on peri-tumour endothelium

A) Low power view of tumour section B) at 2.5x magnification and C) 10x magnification demonstrating positive staining for CD73 on endothelium of peri-tumour vessels (arrows). D and E) Examples of positive staining in peritumoural vessels from two further cases. T = tumour, NT = non tumour.

4.5 Discussion

Analysis of CD73 by immunohistochemistry in a cohort of HCC cases highlights a significantly different expression pattern for this receptor in comparison to cirrhotic and normal liver tissue. Published data in the literature have previously reported an association between increased CD73 expression and reduced survival in HCC.[75] I have used a variety of approaches to quantify CD73 expression in this cohort. The results demonstrate a trend towards poor prognosis, with a positive association between CD73 and vascular invasion, and better overall survival in cases with low percentage of CD73 expression in the tumour, although these findings did not reach statistical significance. Interestingly, when intensity of CD73 was quantified as a percentage, a trend towards worst survival was in the CD73 'equal' group. This cohort could either represent cases without any change in CD73 expression compared to health, or patients where CD73 expression has altered globally throughout the liver, possibly increased in non-tumour and tumour alike, providing one explanation for the reduction in overall survival.

Ma et al. report significant reduction in survival for high CD73 expression in HCC's resected from 189 patients, although the criteria used to determine whether CD73 expression was high or low was not published.[75] Sciarra et al. also report worse overall survival with high expression of CD73 on tumour cells, but in a heterogeneous group comprised of different hepatobiliary cancers, and not specific to HCC alone.[74] The absence of an association between survival and CD73 in the data presented here may be due in part to the patient cohort, which included explanted livers from patients who had undergone liver transplantation. Transplant is the only curative treatment for HCC, and therefore overall

survival after surgery is expected to be higher in this group compared to resection. In addition, patients with a high tumour burden or metastatic disease are unsuitable for surgical treatment and therefore cancers with an aggressive tumour phenotype were less likely to be included in the cohort studied.

CD73 expression on the hepatocyte membrane and within the cytoplasm was a clear distinguishing feature of HCC tumours compared to NL and CLD. The same pattern of distribution has been reported in a small HCC sample (n=24).[74] Intracellular CD73 was shown to be recirculated between the cell membrane and an intracytoplasmic pool in rat hepatocytes.[90] A similar process may explain the findings in the human HCC specimens used in my study, as cases with cytoplasmic staining were associated with strong membranous expression. The presence of CD73 in the cytoplasm may reflect increased protein transcription by the tumour cell to a level that is detectable by IHC. It could also indicate a change in protein function. Mislocalisation of proteins in cancer cells can lead to altered function that promotes tumourigenesis; for example APC (adenomatous polyposis coli) changes location from the cell membrane to cytoplasm in colorectal cancer, leading to loss of tumour suppressor function.[91] Thus, the difference in CD73 expression in cancer cells may represent a functional contribution of CD73 to oncogenesis outside the ATP/ADP/adenosine pathway.

In the future, it would be interesting to assess CD73 in more advanced cases, for example in tumour biopsy specimens from those due to receive systemic therapy to treat large, unresectable tumours or metastatic disease. Although there is less tissue yield from a

biopsy compared to whole tumour resection, this option would expand the extent of cancer stage that was being studied. Furthermore, the fact that CD73 has a circulating form and has also been described on the surface of circulating immune cells suggests alternative approaches could be used to test the biomarker and prognostic properties of CD73 rather than tissue samples.[69] [92] A potential future study could use blood samples to measure CD73 in serum and on immune cells, with comparison between healthy individuals, cirrhosis and HCC.

Table 7 IHC grading and clinical data for 99 HCC tumours stained for CD73

	Sample number	Membranous 1 = yes	Sinusoid grade	Gender	Age (years)	Aetiology	Grade of differentiation	Vascular invasion	AFP (IU/mL)	Survival	Follow up (days)
1	242183	1	3	1	59	ALD	2	1	70	Died	1595
2	249543	1	2	2	67	HBV	2	0	9	Alive	2463
3	249532	1	3	1	67	ALD	2	1	4	Alive	2625
4	249582	1	1	1	61	ALD	3	1	4323	Died	864
5	249556	0	1	1	59	HCV	2	1	3	Died	1135
6	249462	0	2	1	70	Non-cirrhotic	2	1	2	Alive	2128
7	249519	0	2	1	66	HBV	1	0	3	Alive	2570
8	249498	1	3	1	51	ALD	2	0		Alive	2564
9	249471	0	1	1	63	HCV	2	1	3	Died	262
10	249485	1	1	1	53	NRH	1	0	2	Alive	2777
11	254754	0	3	1	66	HCV	2	0	2	Alive	1756
12	242207	0	0	1	66	ALD		0	4	Died	45
13	254694	0	2	1	58	ALD	1	1	15	Alive	2023
14	249568	1	3	1	64	HH	2	1	45	Died	407
15	254706	0	2	2	66	NASH, ALD	2	1	12	Alive	1558
16	254683	0	1	1	80	Non-cirrhotic	2	1	124	Died	100
17	254597	0	3	2	54	NASH	1	0	3	Alive	2283
18	254586	0	1	1	68	ALD	2	1	2	Alive	967
19	242254	0	1	1	62	HCV	2	1	171	Died	2276
20	242170	0	1	1	71	PSC	1	0	4	Died	2326
21	242220	1	3	1	70	ALD	2	1	7	Died	2301
22	254685	0	3	1	58	ALD	1	1	15	Alive	2023
23	275468	0	3	1	54	HCV/ETOH	3	1	45	Died	907
24	254609	0	1	1	63	AIH	2	1	4	Alive	1942
25	254566	0	1	1	54	ALD, NASH	1	0	<1	Died	2077
26	254731	0	1	1	56	HCV	2	1	135	Alive	1933
27	242159	1	2	1	58	HCV	2	1		Alive	3053
28	254734	0	1	2	50	NASH	3	0	2	Alive	2052

	Sample number	Membranous 1 = yes	Sinusoid grade	Gender	Age (years)	Aetiology	Grade of differentiation	Vascular invasion	AFP (IU/mL)	Survival	Follow up (days)
29	275477	0	1	1	62	HCV	2	0	13	Alive	1363
30	254554	0	0	2	62	Non-cirrhotic	2	1		Alive	2266
31	242230	0	1	1	39	HBV	2	1	7	Alive	2750
32	275434	0	1	1	83	NASH	2	0	100	Alive	671
33	254469	0	1	2	47	Cryptogenic	2	1	139	Alive	2382
34	254541	0	1	1	58	HCV	2	1	3	Alive	2292
35	254530	0	1	1	80		2	1	5	Alive	2275
36	254518	1	3	2	57	HCV	2	1	296	Died	145
37	275444	0	2	1	58	ALD	2	1	158	Died	576
38	242194	0	1	1	76	Non-cirrhotic	1	0		Died	1408
39	254482	0	2	1	78	Non-cirrhotic	2	1	94	Alive	1921
40	254457	1	3	1	72	Cryptogenic	2	1	34	Died	47
41	242242	0	2	1	62	ALD	2	1	3	Died	1093
42	254433	1	2	2	39	Non-cirrhotic	2	0	195	Died	2164
43	254446	1	2	1	68	ALD	2	1	6	Alive	2406
44	254506	0	1	1	78	NASH	2	0	5	Alive	747
45	254494	0	2	2	39	Cryptogenic	2	0	63445	Alive	2176
46	242267	1	1	1	61	ALD	2	1		Died	375
47	242146	1	1	1	61	HCV	1	0	77	Died	362
48	242289	1	3	2	53	HCV	2	0	51	Alive	2822
49	242279	0	1	2	65	Non-cirrhotic	3	0		Alive	1570
50	242337	1	3	2	74	NASH	2	1	1220	Died	418
51	242349	0	2	2	70	ALD	1	1		Alive	644
52	249615	0	1	1	83	Non-cirrhotic	1	0	1	Died	2747
53	242327	0	2	1	70	HH	2	0	7	Died	4
54	249712	1	1	2	73	Non-cirrhotic	3	1	2414	Alive	2207
55	249700	0	1	1	66	HCV	3	1	14	Died	18
56	249633	0	1	2	66	PBC	2	0	49	Died	989
57	242373	0	2	1	74	NASH.	1	0		Alive	2848
58	249652	0	1	1	65	ALD	3	1	6828	Died	943

	Sample number	Membranous 1 = yes	Sinusoid grade	Gender	Age (years)	Aetiology	Grade of differentiation	Vascular invasion	AFP (IU/mL)	Survival	Follow up (days)
59	249736	1	2	1	56	HCV, ALD	2	1	26	Alive	2281
60	249675	1	2	1	55	HCV	2	1	9	Alive	2423
61	249688	1	2	1	61	HCV	1	1	112	Alive	2475
62	249628	0	2	2	66	PBC	2	0	49	Died	989
63	249639	1	3	1	54	HCV	1	0	3	Died	1035
64	249591	1	3	1	66	Non-cirrhotic	2	1		Died	503
65	254637	1	2	1	58	ALD	2	1	34	Alive	2172
66	254662	0	1	2	77	Non-cirrhotic	3	0	35815	Alive	2155
67	265163	1	1	1	70	ALD	2	1		Died	1676
68	265223	0	2	1	69	Cryptogenic	1	0		Died	236
69	265259	1	1	2	76	PBC	3	1		Died	1549
70	265199	0	2	1	85	Cryptogenic	1	1	2	Died	1039
71	265236	0	1	2	71	NASH	2	0		Died	2505
72	265188	0	2	2	46	Non-cirrhotic	1	0		Died	1122
73	265251	1	2	1	65	ALD	2	1		Died	1415
74	254649	1	2	1	55	HCV	2	0	33	Alive	2169
75	265151	0	4	1	53	HCV	1	1		Died	834
76	254626	1	3	2	54	HCV/ETOH	1	0	8	Alive	1189
77	265176	1	2	1		HH, NASH	1	1		Alive	2042
78	254616	1	3	2	70	PBC	2	1	310	Alive	2291
79	254421	1	3	1	65	PBC	2	0		Alive	2318
80	254410	0	2	1	75	Non-cirrhotic	2	0	2	Died	738
81	275541	0	2	1	57	HCV		1	21	Died	564
82	275480	0	3	1	56	Non-cirrhotic	2	0	113	Died	758
83	242301	0	2	1	61	HCV, NASH	2	1	5	Alive	2878
84	249604	1	2	1	49	HBV	3	1	<1	Died	199
85	242313	0	2	1	60	ALD	2	0	6	Alive	2813
86	275529	1	3	1	38	HBV	2	1	37	Alive	1687
87	254362	0	0	1	43	HBV	2	1	7	Died	327
88	254375	0	2	1	57	Cryptogenic	2	1	5	Died	1

	Sample number	Membranous 1 = yes	Sinusoid grade	Gender	Age (years)	Aetiology	Grade of differentiation	Vascular invasion	AFP (IU/mL)	Survival	Follow up (days)
89	275506	1	3	1	66	AIH	3	1	3	Alive	1797
90	275552	0	1	1	47	HCV	2	1	2	Alive	2750
91	275493	1	3	1	58	HCV	2	1	57	Alive	2750
92	254397	0	2	1	69	HCV, ALD.	2	1	1746	Died	307
93	254386	1	2	1	62	ALD	2	1	2	Alive	347
94	242363	1	2	No clinical data							
95	265212	0	2	No clinical data							
96	265139	1	2	No clinical data							
97	265128	1	2	No clinical data							
98	275516	0	1	No clinical data							
99	254710	0	1	Tumour compressed against liver capsule							

Membranous staining, 0 = absent, 1 = present; Sinusoidal grade, 1 = tumour less than non-tumour, 2 = equal, 3 = tumour greater than non-tumour; Gender, male = 1, female = 2; Aetiology, alcohol related liver disease (ArLD), autoimmune hepatitis (AIH), hepatitis B (HBV), hepatitis C (HCV), hereditary haemochromatosis (HH), non-alcoholic steatohepatitis (NASH), primary biliary cholangitis (PBC); Grade of differentiation, 1 = well differentiated, 2 = moderate, 3 = poorly differentiated; Vascular invasion 0 = absent, 1 = present; AFP (alpha fetoprotein). Sample no. 99 was excluded from sinusoidal staining analysis due to the location directly under the liver capsule, compressing the non-tumour tissue and confounding the results.

Chapter 5 SPLICED VARIANTS OF CD73 IN CHRONIC LIVER DISEASE AND HCC

5.1 Introduction

In eukaryotes, DNA is transcribed into pre-mRNA (messenger RNA) from which introns are removed and exons spliced together to produce mRNA for protein synthesis. Carefully regulated variation in which exons are included in pre-mRNA leads to the transcription of different proteins from the same gene.[93] This process is termed alternative splicing, and helps to maintain normal cell function and homeostasis.[94] However, in cancer, some spliced variants are specific to tumour cells and can promote oncogenesis.[95] For example, a variant androgen receptor in prostate cancer is associated with cancer cell growth following anti-androgen treatment, leading to reduced overall survival.[96] Spliced variants have also been shown to contribute to tumour development in HCC, such as the TLL1 gene in Hepatitis C related HCC.[97] Snider et al. report a spliced variant of NT5E (CD73) which lacks exon 7. This short variant was found to be upregulated in Hepatitis C related cirrhosis and HCC, and encoded a protein that was structurally and functionally distinct from canonical CD73, lacking nucleotidase activity.[80] I wanted to explore the potential role of spliced variants of CD73, and therefore performed qPCR to measure gene expression of canonical CD73 and the spliced variant in normal human liver, cirrhosis and HCC, and primary human hepatic sinusoidal endothelial cells (HSEC).

I also performed immunofluorescence on isolated HSEC, with a view to addressing the final aim of this project. HSEC were available through cryopreserved stocks from the liver laboratory team. HSEC were isolated from whole liver tissue by an established technique using density gradient centrifugation and magnetic bead separation. Previous studies have confirmed that these cells maintain their phenotype up to passage 4-5 and after

cryopreservation.[47] The cells were maintained in culture with media supplemented by vascular endothelial growth factor and hepatocyte growth factor.

Aim 4: To study the expression of CD73 in primary human hepatic sinusoidal endothelial cells and its regulation by factors found in the inflammatory/tumour microenvironment.

5.2 CD73 is expressed by isolated primary human HSEC and the expression pattern is altered by pro-inflammatory cytokine stimulation.

Using immunofluorescent staining, the expression of CD73 was detected on isolated HSEC. CD31 is expressed on the HSEC membrane and was used as a positive control, and to aid definition of the cell membrane. There was positive staining for CD73 within the cytoplasmic compartment, although it did not co-localise with CD31. Following stimulation with a combination of the pro-inflammatory cytokines interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα), there appeared to be a change in the expression pattern of CD73 with significant nuclear staining (Figure 5-1).

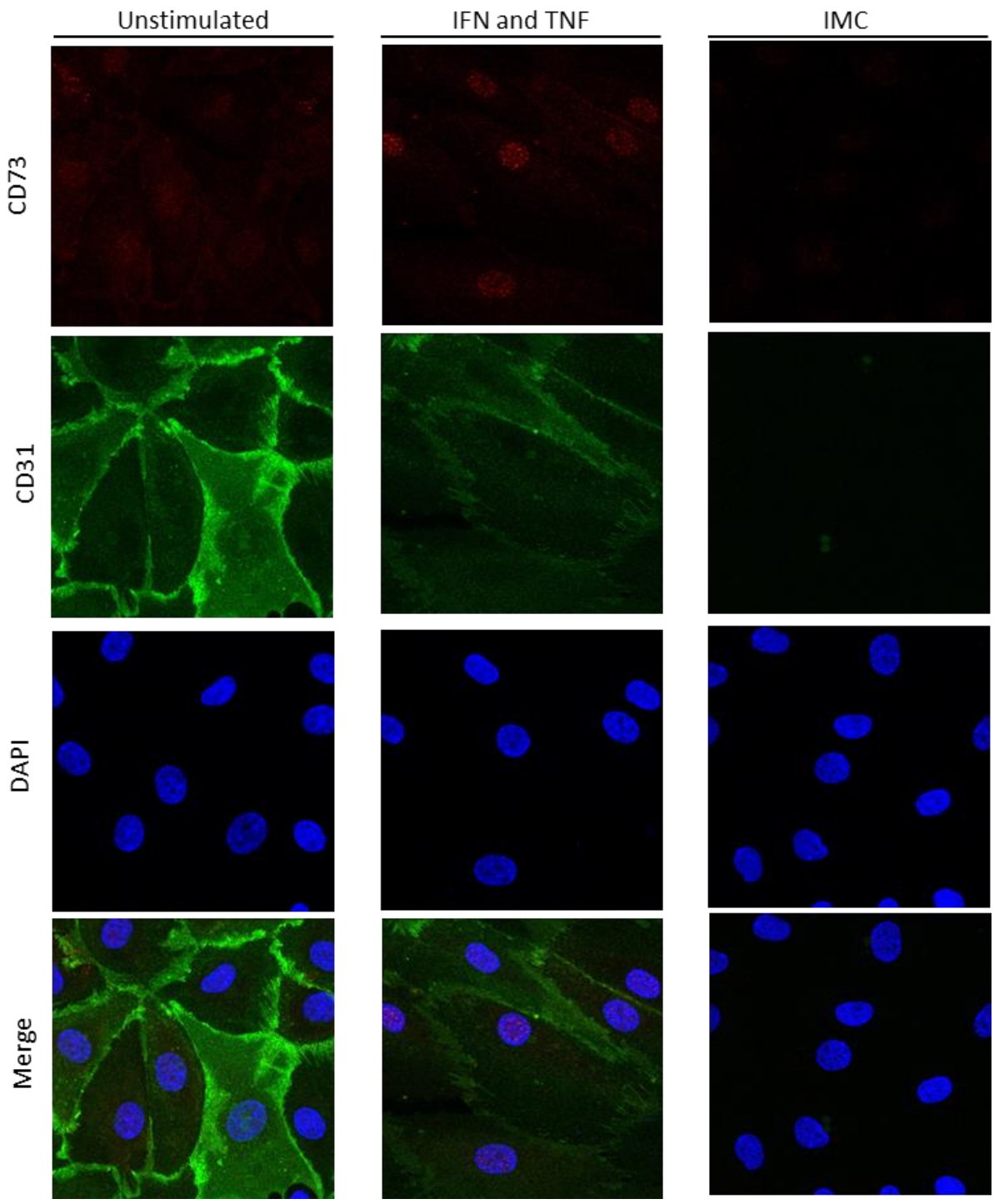


Figure 5-1 CD73 is expressed in the cytoplasm of cultured HSEC, and in HSEC nuclei following stimulation with TNFα and IFNγ

Immunofluorescence labelling for CD73 in cultured HSEC. CD73 (red) is positive in HSEC cytoplasm. CD31 (green) is positive on the HSEC membrane. HSEC treated with TNFα and IFNγ also express CD73 in the nuclei, which co-localises with DAPI (blue). IMC = Isotype matched control.

5.2 Gene expression of the CD73 spliced variant is low in chronic liver disease

Following the confirmation that CD73 was expressed at the protein level in both whole liver tissue and in isolated primary HSEC, I proceeded to study its regulation at the transcript level. I initially performed quantitative PCR on whole liver tissue to establish whether the short variant is detectable at the transcript level, as the CD73 antibody epitope is present in the full-length protein and the spliced variant, and therefore the antibody does not discriminate between the isoforms on tissue. RT-PCR confirmed mRNA expression of the canonical CD73 gene, hereafter referred to as CD73L, and the spliced variant, CD73S (determined relative to that of 18S) in NL and CLD. A third primer contained both forms of the transcript, referred to as CD73combined (CD73comb). CD73L and CD73comb were present in NL and all forms of CLD. CD73S was expressed at low levels in NL and CLD (Figure 5-2).

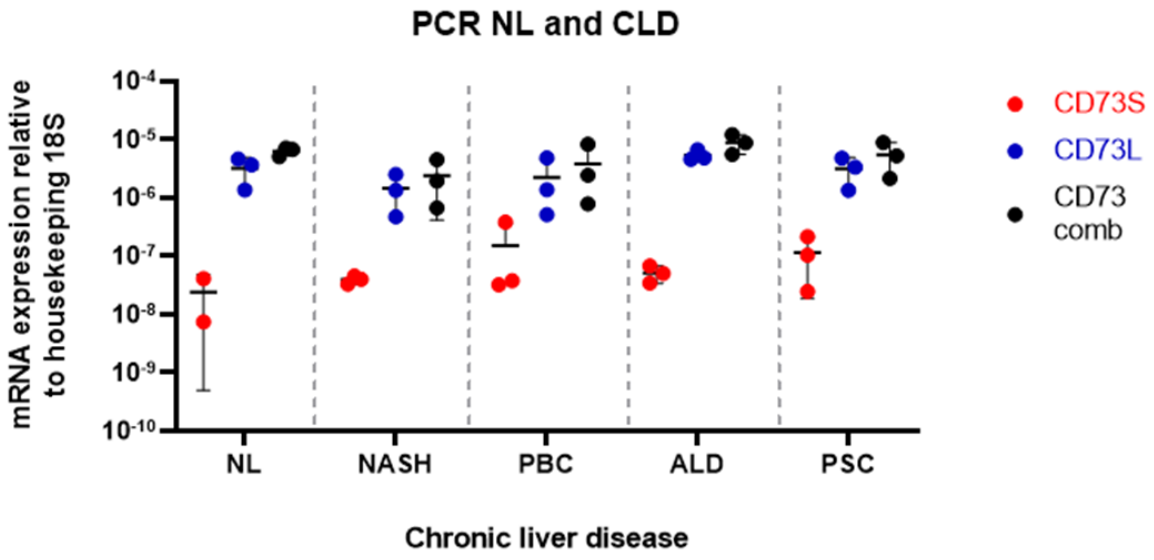


Figure 5-2 CD73L and CD73comb are expressed in NL and CLD. CD73S is expressed at low levels in CLD.

Mean mRNA expression of CD73 on whole liver tissue relative to the housekeeping gene 18S. N=3 with each sample tested in triplicate. Horizontal line denotes mean with standard deviation. NASH = non-alcoholic steatohepatitis, PBC = primary biliary cholangitis, ALD = alcohol related liver disease, PSC = primary sclerosing cholangitis.

5.3 Gene expression of CD73 is increased in HSEC compared to Huh7 and HepG2

After studying gene regulation in whole liver tissue, I proceeded to analyse the expression in separate populations of liver cells, focusing on hepatocytes (epithelial cells of the liver) and HSEC. Primary hepatocytes are challenging to isolate and do not survive in culture and therefore I used Huh7 and HepG2, immortalised cell lines from well differentiated HCC tumours. PCR for canonical and spliced variants of CD73 in isolated HSEC and the epithelial cells lines revealed upregulation of CD73 gene expression in mRNA from HSEC compared to Huh7 and HepG2 (relative to housekeeping gene GAPDH) (Figure 5-3).

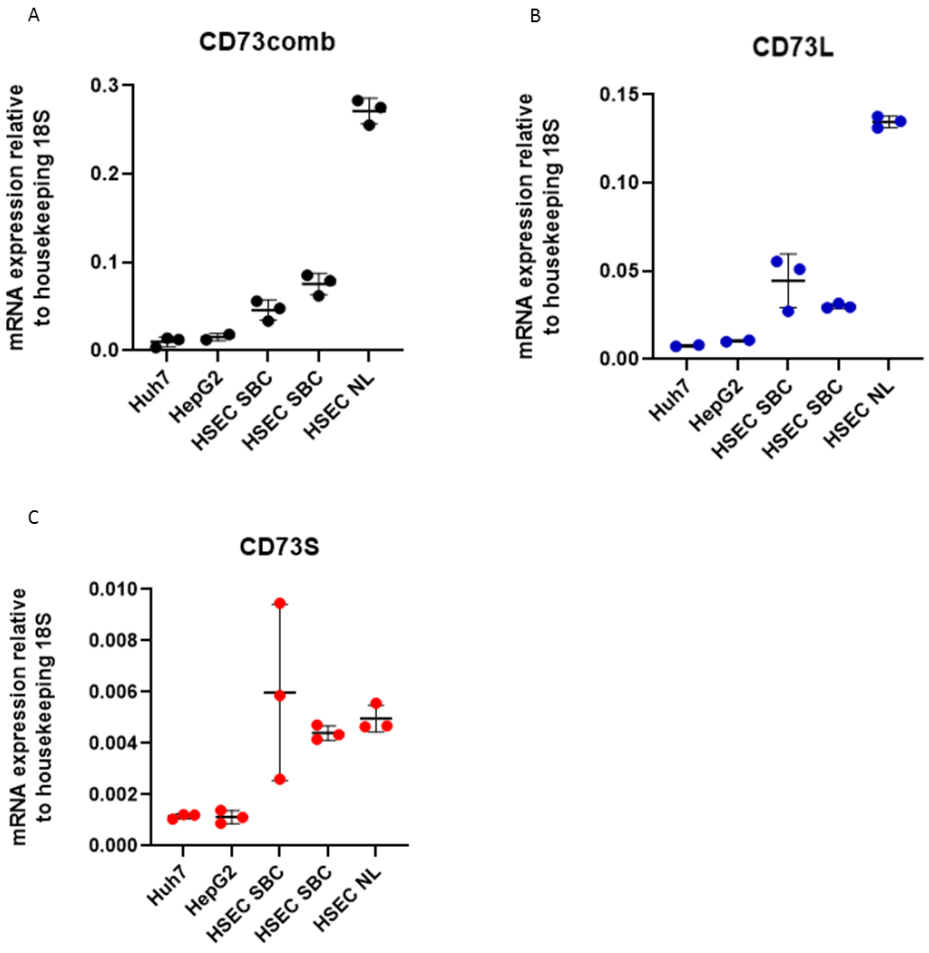


Figure 5-3 CD73 gene expression is upregulated in HSEC compared to cancer cell lines Huh7 and HepG2

Gene expression for CD73 is greater in HSEC (N = 3) compared to Huh7 and HepG2 (N=1) for A) the canonical form, B) the combined form and C) the spliced variant. Each sample tested in triplicate. SBC = secondary biliary cirrhosis, NL = normal liver. Horizontal line denotes mean with standard deviation.

5.4 CD73 expression in HSEC is not increased by shear stress

The vascularity of HCC tumours changes compared to normal or cirrhotic liver parenchyma. Cancerous lesions become arterialised and demonstrate 'washout' on contrast imaging. These pathological features are specific for HCC and therefore used as diagnostic criteria. We have replicated this environment *in vitro* by exposing isolated HSEC to 1Pa shear stress before extracting cDNA, provided by Alex Wilkinson, a PhD student in the Shetty group. PCR on HSEC did not show a difference between static and shear stress conditions for any of the transcripts studied (Figure 5-4).

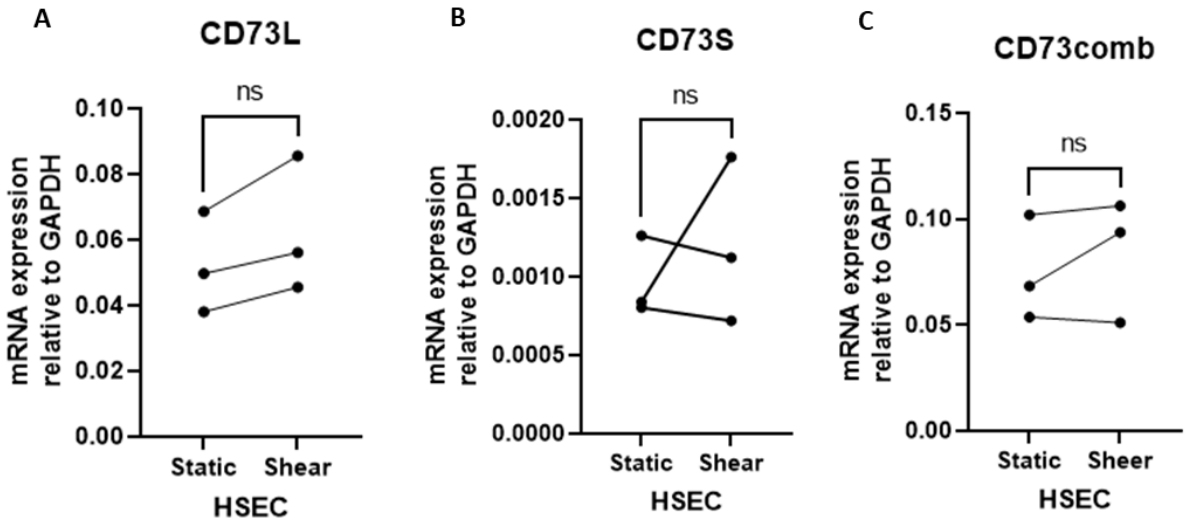


Figure 5-4 CD73 expression on HSEC is not affected by shear stress

PCR for HSEC in static and 1Pa shear stress conditions for 72 hours did not show any change in gene expression of A) CD73L, B) CD73S or C) CD73comb using paired t-test. N=3, each sample tested in triplicate.

5.5 Discussion

The results from chapters 3 and 5 confirm that CD73 is expressed on sinusoids in tissue and maintains expression in isolated HSEC. I went on to study the effect of pro-inflammatory cytokines on CD73 expression, choosing TNF-α and IFN-γ because of their established contribution to chronic liver disease.[98] [99] Interestingly, localisation of CD73 is affected

by pro-inflammatory cytokines to include the HSEC nucleus. Although, in the context of previous published literature, expression in the nucleoplasm has also been shown by IF on a skeletal myocyte cell line, CD73 is widely described as a cell surface protein and its position in the nucleus is not established.[51] A Western blot on membrane, cytoplasm and nuclear fractions could be performed to evaluate whether the result is due to non-specific binding.

IFN-γ does not increase CD73 expression in isolated human cells from cardiac atrium, and has been shown to reduce transcription and activity of CD73 in astrocytes.[100] Conversely, TNF-α increases CD73 protein expression and activity in astrocytes without increasing mRNA, which could result from mobilisation of intracellular stores.[101] CD73 expression is also increased on melanoma cells in mice stimulated by TNF-α and hepatocyte growth factor [102]. To date, I am not aware of any reports in the literature regarding the effect of IFN-γ and TNF-α on CD73 expression on HSEC or other hepatic cellular populations. It would be interesting to explore this further, initially by assessing HSEC stimulated with a single factor only, to observe if there is a difference in effect between IFN-γ and TNF-α.

Regarding the evaluation of spliced variants of CD73, NL and CLD primarily expressed the canonical transcript, CD73L, with low level expression of the spliced variant. In respect to CD73 gene expression in HCC, malignant hepatocytes are difficult to maintain in culture, hence the use of hepatoma cell lines for these experiments. I found increased gene expression of CD73S in Huh7 compared to HSEC, which may account for the membranous expression on hepatocytes in tumour samples. Furthermore, the cytoplasmic staining of CD73 seen in tumour hepatocytes only may represent intracellular expression of CD73S. As

reported by Snider et al, CD73S does not have nucleotidase activity, is mainly intracellular and is upregulated in HCC, suggesting that CD73 may have pro-tumourigenic effects beyond the immunosuppressive action of the CD39/CD73/adenosine pathway.[80] Future study could include staining Huh7 to assess where CD73 is located within the cell and in situ hybridisation to analyse which transcripts are being coded.

HSEC also expressed the spliced variant, at half the level of the hepatoma cell line. It would be interesting to compare expression in HSEC from NL and CLD, to see if cirrhosis affects gene expression. Future studies could also compare HSEC with other vascular endothelium, such as HUVEC (human umbilical vein endothelial cells) and lymphatic endothelium. Lymphatics are more similar to HSEC than vascular endothelium, and do express CD73 [103]. Whether this includes the spliced variant, or if the level of expression is different in liver sinusoids, has not been established.

My experiments focused on the gene expression of CD73 splice variants, studying this in both whole tissue and cultured cells. The next step would be to distinguish the location and/or cell type that is expressing the protein. Immunofluorescence (IF) staining has confirmed CD73 expression in the cytoplasm and cell membrane of HSEC. It would be interesting to repeat IF for CD73 in HSEC exposed to pro-inflammatory cytokines such as TNFα and IFNγ and other cytokines that are relevant to liver disease, such as TGF-β. Western blot could be performed on treated HSEC to determine whether the spliced protein is present, and if its expression is influenced by these factors.

In chapter 4, I have confirmed that CD73 is universally expressed on endothelium of vessels surrounding HCC tumours using IHC. The tumour microenvironment is known to be characterised by altered shear stress, in addition to which HCC is a particularly hypervascular tumour. Previous studies have shown that endothelial CD73 is regulated by shear stress, with increasing shear stress promoting the activity of CD73.[104] I therefore undertook studies of CD73 gene expression in HSEC exposed to low and high shear stress. Although shear stress did not significantly alter endothelial expression of CD73 in HSEC in my initial studies, future experiments could combine the effect of altered blood flow and other factors to better replicate the tumour environment in HCC, such as shear stress and pro-inflammatory cytokines or hypoxia.

Chapter 6 DISCUSSION

6.1 Unmet needs in HCC

HCC is a global health problem. Despite breakthroughs in cancer treatment over the last ten years, mortality from liver cancer is increasing. Whilst the vast majority of hepatocellular tumours develop in the context of liver cirrhosis, the risk varies according to the aetiology of the underlying chronic disease. Incidence of HCC in alcohol related liver disease is around 8%, compared to up to 30% in hepatitis C.[105] There remains a lack of understanding of which additional factors contribute to tumour development, made evident by the limited success of treatments that are currently available for HCC. The poor response to immunotherapy in HCC arising in the context of NASH compared to chronic viral hepatitis again serves to highlight the different mechanisms of tumourigenesis in HCC, which extend beyond a simple paradigm of scarring and fibrosis.[106] As such, there is a significant unmet need in our understanding of which patients with liver disease will develop HCC, and why.

The role of tumour vasculature is one aspect of pathogenesis that warrants further study. As HCC tumours are characterised by their highly vascularised nature, it is reasonable to expect that agents targeting angiogenesis would produce a significant response, but this approach has historically shown limited effect. After a series of failed clinical trials, the tyrosine kinase inhibitor Sorafenib became the first anti-angiogenic treatment to show modest success through targeting VEGF (vascular endothelial growth factor) and PDGF (platelet derived growth factor).[24] Although the average survival benefit from Sorafenib is just three months, it remained the only systemic treatment available for HCC for over a decade. However, the recent IMbrave150 trial has shown good response to the anti-PD-1 immunotherapy agent Atezolizumab, which was augmented by combination with

Bevacizumab, a VEGF inhibitor.[18] This highlights the importance of the tumour vasculature in HCC and paves the way for other vascular targets to be investigated in combination therapy.

6.2 CD73 as a potential vascular therapeutic target for HCC

Liver sinusoids are the gatekeepers to hepatic immunity, and preliminary data from the Shetty group has interestingly shown how liver endothelia actively alter transcription of immune mediated pathways in HCC. NT5E is one such pathway that is upregulated in hepatocellular tumour endothelium. It codes for a protein that exerts an immunosuppressive effect via production of extracellular adenosine. I have shown that CD73 is expressed on sinusoids in normal liver and chronic disease, and that expression in the sinusoids varies in HCC. The distribution of CD73 is also different in HCC, whereby tumour hepatocytes can have positive staining on the entire cell membrane, and in some cases, in the cytoplasm. Low expression of CD73 in the tumour compared to matched non-tumour tissue was associated with a trend towards improved overall survival. Although the results did not reach statistical significance, they show a trend that associates CD73 with a poor prognosis, which is consistent with published literature. The presented results also validate the RNA sequencing data by confirming that CD73 is increased in HCC at the protein level.

Two functional proteins are transcribed from the NT5E gene. I have shown that the shorter form was minimally expressed in normal liver tissue and chronic disease at the transcript level. Gene expression of CD73 is increased in HSEC compared to the cancer cell lines Huh7

and HepG2, but this was across all primers for CD73 and not specific to the spliced variant. This is consistent with the hypothesis that CD73 promotes an immunosuppressive tumour microenvironment through its enzymatic activity, as the short form is catalytically inactive and therefore is not expected to contribute to adenosine production.[80]

The critical finding from a vascular perspective is that in all the HCC cases the peri-tumoural vessels in the tumour capsule were positive for CD73. It is therefore possible that CD73 plays an important role in the immunosuppressive tumour microenvironment of HCC. I have also confirmed that HSEC maintain their expression of CD73 *in vitro.* This provides the capability to study the functional contribution of CD73, as well as its separate splice variants, in the vascular biology of the liver.

6.3 Future Considerations

Whilst I have tested CD73 in a range of disease states, both in chronic liver disease and HCC, it would be interesting to look specifically at expression in viral hepatitis, and compare it to other conditions such as NASH. Additionally, serum CD73 could be measured to establish whether circulating levels acts as potential biomarkers and correlate with tumour characteristics or prognostic factors, and if they change in response to HCC treatment.

Further work in tumour samples could explore the relationship between CD73 and immune cells, initially by co-staining tumour sections for CD73 and markers of immune activation. Theses could include markers of tumour promoting cells, such as tumour associated

macrophages or FoxP3 for regulatory T cells, or CD56 and CD8 for anti-tumour NK cells and cytotoxic T effector cells respectively.

For future *in vitro* studies, HSEC could be stimulated with pro-inflammatory cytokines such TNFα in combination with other factors associated with the tumour microenvironment, such as shear stress or LPS, to test for an effect on CD73 expression.

It would also be interesting to examine the functional effect of CD73 and whether modulating it affects the microenvironment. For example, using siRNA knockdown or inhibiting CD73 function on HSEC using a blocking antibody, and measuring the effect on immune cell recruitment using a flow assay. The interaction between tumour and endothelium could also be explored by measuring CD73 gene and protein expression in HSEC co-cultured with Huh7, or in work with mouse models of HCC. Viable CD73 knockout animals have previously been generated and have already been studied in the context of glioblastoma, demonstrating increased response to immunotherapy in CD73 knockout compared to controls.[107] It is therefore feasible to explore the role of CD73 in murine models of HCC, however, generating HCC animal models that reflect what is observed clinically in humans is currently proving challenging. It is imperative that any potential future studies of CD73 in animal models of HCC accurately recapitulate the inflammatory and fibrotic tumour microenvironment, since we hypothesise that CD73 on surrounding blood vessels within the tumour capsule make an important contribution to the progression of human HCC, rather than intra-tumoural expression.

Therefore, there are a number of avenues to pursue to further our understanding of CD73 in liver cancer, with a hope it could be a potential therapeutic target to combat the immunosuppressive tumour microenvironment of HCC.

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Appendix

CD73 as a key endothelial receptor driving the immunosuppressive HCC microenvironment

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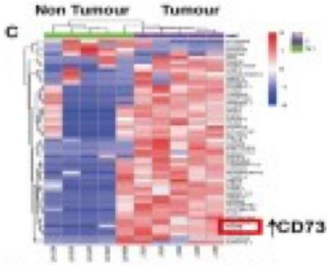
Introduction: Immunotherapy has transformed the prognosis of many cancers, yet results in hepatocellular cancer (HCC) are limited to a subgroup of patients. Identifying immunosuppressive endothelial factors in the tumour microenvironment (TME) is critical to improve outcomes for HCC. CD73 is an ectonucleotidase that converts AMP to adenosine to suppress T -cell activity.

Aim: To explore the expression of CD73 in HCC as a potential contributing factor to an immunosuppressive TME.

Methods: RNA sequencing (RNAseq) was performed on endothelium isolated from human HCC and matched non -tumour endothelial cells from the same patient (Fig 1). To validate the findings, we undertook immunohistochemistry for CD73 in 50 samples of human HCC, and the pattern of CD73 expression in tumour and adjacent non -tumour was compared to clinical outcomes. Immunofluorescence for CD73 was performed on isolated liver sinusoidal endothelial cells (LSEC).

Figure 1. Tumours reprogramme liver endothelium to prevent immune eradication

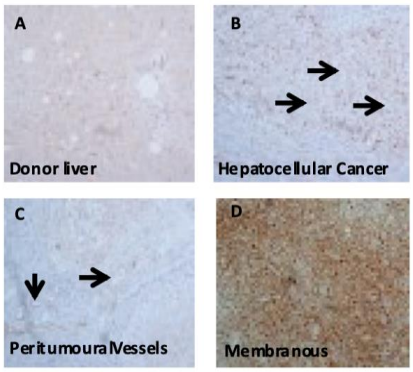


Ulex lectin beads were used to selectively isolate endothelium from tumour and matched non-tumour tissue from 5 HCC patients. RNA was extracted and sent for deep sequencing. Heat map analysis demonstrated significant upregulation of immunomodulatory genes including CD73

Results: RNAseq data demonstrated that several genes were significantly upregulated on tumour endothelium, including CD73 (Fig 1). Immunohistochemistry for CD73 on 50 HCC sections confirmed that the protein is present on vascular endothelium and in HCC tumours (Fig 2). The pattern of expression was different in tumour compared to matched non -tumour control of the same patient, with a membranous staining pattern in tumour. Increased tumour expression of CD73 correlated with reduced overall survival (p=0.73) (Fig 3).

We also confirmed cell membrane and cytoplasmic expression of CD73 in cultured primary human LSEC using dual colour immunofluorescence (Fig 4).

Figure 2. CD73 is upregulated within tumours and surrounding blood vessels compared to non-tumour tissue



Immunohistochemistry for CD73 was undertaken of paraffin embedded sections of A) donor liver and B) HCC. Arrows highlight CD73 expression on tumour sinusoidal channels, C) vasculature surrounding tumour and D) membranous staining of hepatocytes

Figure 3. Kaplan Meier curve of survival after liver cancer resection or transplant, by high or low tumour expression of CD73

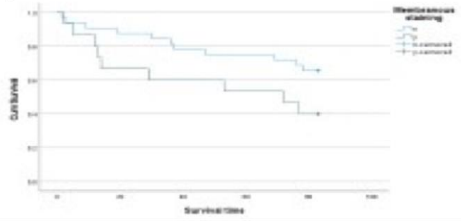
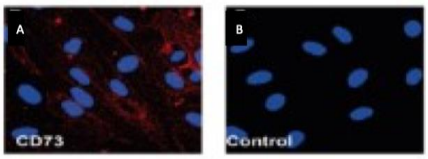


Figure 4. Isolated primary human liver endothelial cells maintain CD73 expression in culture



A) Immunofluorescent images of isolated primary liver endothelial cells stained with CD73 antibody and B) images of staining with isotype matched control

Conclusion: RNA seq results demonstrate upregulation of CD73 in HCC tumour endothelium . We have validated this at the protein level using immunohistochemistry and detected CD73 on isolated LSEC. Increased CD73 expression was associated with reduced overall survival. Endothelial CD73 may contribute to the immune HCC microenvironment through adenosine -mediated T -cell suppression, making it a promising target for immunotherapy.