School of Medicine

Ysgol Meddygaeth

Immune Fingerprinting of Bacterial Infections in Decompensated Cirrhosis

Master of Philosophy (Infection and Immunity)

August 2022

Emily Fflur Jacob

Abstract

Background and Aims

Decompensated cirrhosis is associated with an increased risk of infection. Acute-on-chronic liver failure (ACLF) is a severe phenotype associated with immune dysregulation. Immunological phenotypes of individuals with advanced liver disease may enable stratification of patients to specific treatments. The humoral profile in peripheral blood at progressive stages of cirrhosis, contribution to prognosis, and risk of complications is yet to be fully defined. Cellular and humoral profiles in peripheral blood (and ascites where possible) of individuals with cirrhosis were investigated to identifying immunological phenotypes of advanced liver disease.

Method

Patients were recruited from the hepatology day case and inpatient service at the University Hospital of Wales. Participants were stratified into healthy donors, no acute illness Child-Pugh (CP)-A, no acute illness CP-B, chronic decompensation, acute decompensation (no infection), acute decompensation (infection) and ACLF. Conventional/non-conventional lymphocytes were analysed by multiparameter flow cytometry and 31 analytes were analysed by Luminex multiplex assays comparing analyte concentrations in peripheral blood and ascites.

Results

ACLF monocytes were significantly elevated compared to no acute illness CP-A, no acute illness CP-B and chronic decompensation no infection. MAIT cells were significantly reduced in no acute illness (CP-B), acute decompensation (no infection) and ACLF patients. Chronic decompensation and ACLF were associated with increased expression of PD1 on ascites $CD4^+$ and $CD8^+$ T cells. Ascites, IL-6, IL-10, VEGF, CXCL10 and CCL2 levels were significantly higher compared to peripheral blood.

Conclusion

Alterations in the monocytic lineage may correlate with disease severity. Reduction in MAIT cells alongside PD1 overexpression, and subsequent T cell exhaustion, may contribute to immune suppression in advanced liver disease. Future work will apply non-directed clustering as a tool to better stratify patients based on complex immune signatures.

Table of Contents

List of Figures

List of Tables

Abbreviations

Abbreviation	Definition
ACLF	Acute-on-chronic liver failure
AIH	Autoimmune hepatitis
ALD	Alcoholic liver disease
APC	Antigen-presenting cell
CP	Child-Pugh
CRP	C-reactive protein
DC	Dendritic cell
ECM	Extracellular matrix
EGF	Endothelial growth factor
FMO	Fluorescence minus one
GI	Gastrointestinal
HE	Hepatic encephalopathy
HSC	Hematopoietic stem cells
HCV	Hepatitis C virus
LSEC	Liver sinusoidal endothelial cells
MAIT	Mucosal-associated invariant T
MHC	Major histocompatibility complex
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCT	Non-conventional T
NK	Natural killer
NKT	Natural killer T
PAMP	Pathogen-associated molecular pattern
PDGF	Platelet-derived growth factor
PRR	Pattern-recognition receptor
RAAS	Renin-angiotensin-aldosterone system

R^+	RPMI-1640, penicillin, streptomycin, L-glutamine and 10% fetal calf serum
RBC	Red blood cell
SBP	Spontaneous bacterial peritonitis
sICAM-1	Soluble ICAM-1
SNS	Sympathetic nervous system
sPD-L1	Soluble PD-L1
TCR	T cell receptor
T_H	Helper T
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
WCC	White cell count

Acknowledgments

I would like to thank my MPhil supervisors Dr. Thomas Pembroke and Prof. Matthias Eberl for giving me the opportunity to work on this project and for their knowledge, guidance and encouragement throughout my two years at Cardiff University. By undertaking this MPhil, I have gained invaluable skills and experiences working autonomously in a laboratory and has been a great platform for my transition onto a clinical role.

Further, I would like to thank Prof. Bernhard Moser, Dr. Simone Cuff, Ross Burton and Sarah Baker for their enthusiasm for the project and continued guidance and advice throughout the weekly laboratory meetings. In particular, I am very grateful for Sarah Baker for her knowledge and involvement in Luminex experiments and educating me to become competent using novel equipment.

I am extremely grateful for all healthy donors and patients who participated in the project, and to the nurses on the Hepatology ward, in particular Tara Rees, for their help with patient recruitment and sampling.

On a personal note, I would like to give an extended thanks to my family for their unconditional support and encouragement throughout my education, and particularly during the last two years studying amid the pandemic.

Thank you all.

Word count: 14781

1. Introduction

1.1 Introduction to the Immune System

The immune system consists of numerous effector cells and molecules responsible for the protection against foreign antigens such as bacteria, fungi, viruses and toxins. Immune cells are derived from pluripotent haematopoietic stem cells (HSCs) within the bone marrow. Once the HSC receives specific chemical signals, it divides and differentiates into specific lineages and cell types, as depicted in Figure 1. The immune system is broadly categorised into the innate and adaptive arms. The innate immune system offers a broad and rapid front-line response that is activated within hours. In contrast, the adaptive immune system is activated within days and offers a more specific response accompanied by long-term immunity against the particular antigen encountered (Murphy and Weaver, 2016).

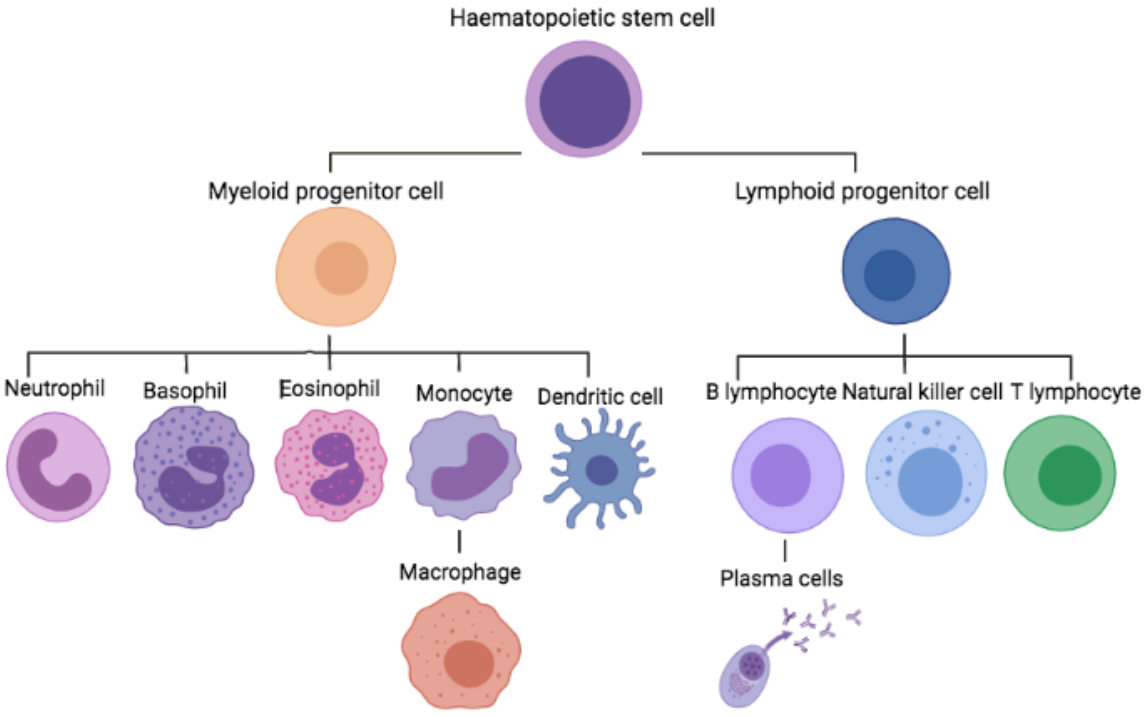

Figure 1 Differentiation of immune cells into different lineages and cell types

Figure adapted from Janeway's Immunology 9th Edition page 4 and created in BioRender.com.

1.2 Innate Immune System

The myeloid lineage gives rise to phagocytic and inflammatory cells of the innate immune system. Cells of the innate arm include phagocytes (neutrophils and macrophages), dendritic cells (DCs), basophils and eosinophils. Phagocytic cells possess pattern recognition receptors (PRRs) that recognise a limited number of conserved components of pathogens such as lipopolysaccharides, peptidoglycan, and double-stranded RNA, termed pathogen-associated molecular patterns (PAMPs). Following recognition, the phagocyte internalises the microorganism into a phagosome. The phagosome fuses with the lysosome, a membrane-bound organelle with contains digestive enzymes and bactericidal products. This creates a hostile environment in order to eradicate the pathogen. DCs also phagocytose microorganisms. However, unlike neutrophils, DCs and macrophages display antigenic fragments of the engulfed pathogen on their surface for recognition and activation of the adaptive immune system. These cells are termed antigen-presenting cells (APCs) and are crucial for the interplay of the innate and adaptive immune response (Murphy and Weaver, 2016).

Another major role of the innate immune system is the recruitment of immune cells to the site of infection or injury. This is termed an inflammatory response and is achieved by the production of signalling molecules, termed cytokines. The immune system has an extensive number of cytokines produced primarily by tissue cells at the site of infection and immune cells. Cytokines facilitate the further influx of cells in order to amplify the immune response. They bind to receptors expressed on the surface of the target cell which triggers intracellular signalling cascades leading to altered gene expression. This results in the differentiation, activation or proliferation of the target cell. A subset of cytokines, termed chemokines, facilitate the influx of cells by creating a concentration gradient in order to mediate migration of innate immune cells to the source of infection.

1.3 Adaptive Immune System

Adaptive immune cells consist of T and B lymphocytes which are derived from the lymphoid lineage.

Conventional T lymphocytes

Conventional T lymphocytes express αβ T cell receptors (TCR) and recognise peptide antigens presented by major histocompatibility complex (MHC) cells. Each T lymphocyte expresses a TCR with unique antigen specificity, and T lymphocytes that are yet to encounter an antigen are termed "naïve". Naïve T lymphocytes migrate to the secondary lymphoid tissue until they receive a specific signal from a DC. Once the DC has encompassed and endocytosed a pathogen, it migrates to the secondary lymphoid tissue where it displays peptide fragments to naïve T lymphocytes. Lymphocytes with cognate receptors for the antigen presented are activated and migrate to the peripheral tissue to carry out their effector function (Murphy and Weaver, 2016).

There are two classes of T lymphocytes: $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ helper T lymphocytes. Cytotoxic T lymphocytes are responsible for the killing of infected cells via interaction with MHC I-peptide complexes on the cell surface. Upon activation, cytotoxic T lymphocytes release perforins, granzymes and granulysin which synergistically disrupt the cell membrane, killing the infected cell (Murphy and Weaver, 2016).

DCs display peptide fragments on their MHC II molecules in order to activate $CD4^+$ helper T lymphocytes. Depending on the costimulatory signals and cytokines released by the DC, the naïve CD4 T cell can differentiate into different classes of helper T (T_H) cells (Murphy and Weaver, 2016). T_H1 secrete IFN-γ and TNF-α which activate macrophages. This innate response is required for the clearance of external bacteria. T_H2 cells produce IL-4, IL-5, IL-10 and IL-13 which activate B lymphocytes whilst inactivating macrophages and monocytes of the innate system (Murphy and Weaver, 2016). T_H17 cells produce IL-17 to stimulate neutrophil development and chemotaxis during bacterial or fungal infections (Louten, Boniface and de Waal Malefyt, 2009).

Natural killer (NK) cells, defined as $CD56^+$ $CD3^-$ cells, are regarded as cells of the innate immune system although they are derived from the lymphoid lineage. NK cells offer a method of immune surveillance in the circulation by identifying infected cells, lacking MHC class I molecules ("missing self") on their surface (Nicholson, 2016). Similar to cytotoxic T

lymphocytes, NK cells kill the infected cells by releasing lytic granules resulting in apoptosis of the target cell (Nicholson, 2016). However, unlike T cells, NK cells lack receptors that are antigen-specific (Murphy and Weaver, 2016).

Unconventional T Lymphocytes

Unconventional T cells have innate lymphocyte-like properties. Several 'unconventional' lineages of T lymphocytes also develop in the thymus.

Natural Killer T (NKT) cells are a form of unconventional T lymphocyte which recognise lipid antigens presented by CD1d, an MHC class I-like molecule (Pellicci, Koay and Berzins, 2020). NKT cells are classified into type 1 and type 2 NKT cells. Type 1 NKT cells express αβ TCRs with an invariant α-chain and are referred to as invariant NKT cells, whereas type 2 NKT cells (diverse NKT cells) lack the highly conserved TCR α-chain (Pellicci, Koay and Berzins, 2020). A number of ligands have been identified for these cells, including α-galactosylceramide, α-glucuronosylceramides and α-glucosyldiacylglycerol (Godfrey et al., 2015). Following interaction with these lipids, NKT cells rapidly release a number of cytokines, including IFN-γ and IL-4 (Godfrey et al., 2015). Furthermore, these effector cells also upregulate CD40, the receptor for CD40 ligand, which along with the production of cytokines, activates DCs to express costimulatory molecules and express IL-12 (Godfrey et al., 2015). These cells also have a specificity to lipid-based antigens, however type 2 NKT cells are more abundant than type 1 and have broader specificity towards lipid antigens (Pellicci, Koay and Berzins, 2020).

Mucosal-associated invariant T (MAIT) cells express TCRs with an invariant Vα7.2 chain typically paired with Vβ2 or Vβ13 chains and are enriched within the liver (Pellicci, Koay and Berzins, 2020). Non-polymorphic MHC class I-like protein (MR1) is responsible for the development and function of MAIT cells liver (Pellicci, Koay and Berzins, 2020). MAIT cells interact with microbial-derived vitamin B metabolites, including folate (vitamin B9), riboflavin (vitamin B2) and other intermediates derived from the B2 synthesis pathway (Godfrey et al., 2015). Folate derivatives act upon MAIT cells to inhibit their function, whereas riboflavin derivatives stimulate MAIT cell activation (Godfrey et al., 2015).

Another type of unconventional lymphocytes are the γδ T cells which express TCR γ and TCR δ chains and account for 0.5-5% of all circulating T lymphocytes (Pellicci, Koay and Berzins, 2020). In human blood, Vγ9Vδ2 T cells are the most abundant, which respond to phosphoantigens such as HMB-PP produced by most bacteria (Liuzzi et al., 2015) (Godfrey et al., 2015). Furthermore, $TRDV1^{+}$ γδ cells become activated by stress-induced proteins including MHC class-I related chain A and UL16-binding proteins, therefore suggesting that γδ cells are stimulated to deplete infected cells (Godfrey et al., 2015).

B Lymphocytes

B lymphocytes have receptors, termed immunoglobulins, which are antigen specific. Naïve B lymphocytes encounter and internalize an antigen via their surface immunoglobulin. The antigen is delivered to the endosomal compartment of the cell where it is digested into fragments. These fragments are presented on the cell surface bound to MHC class II molecules. This complex is recognised by helper T lymphocytes bearing the receptor complementary to the fragment. This interaction stimulates the B lymphocyte to proliferate and differentiate into plasma cells which secrete antibodies against the specific antigen. Antibodies have various protective mechanisms, for example they can bind directly to the pathogen, opsonising it to promote uptake and killing by phagocytes. In many infection cases, toxins released by the microorganism are central to the disease process. To combat this, antibodies are capable of neutralising the toxin, making the bacteria less virulent or avirulent. Antibodies bound to the pathogen can also result in the fixation of complement and activation of the classical pathway by means of complement-mediated cytotoxicity. The complement system is responsible for triggering an inflammatory response, attracting phagocytes to the site of inflammation and stimulating further antibody production. Another mechanism is antibody-dependent cell-mediated cytotoxicity. Antibodies attach to the bacteria via their Fab domain and are able to bind phagocytes such as macrophages through their Fc domain, allowing the macrophage to engulf and kill the pathogen (Murphy and Weaver, 2016).

1.4 Functions of the Liver

The liver is located on the upper right-hand side of the abdominal cavity and is receives its blood supply from the hepatic artery and portal vein (Figure 2) and is drained by the hepatic

vein. It is the largest internal organ and plays a major role in protein synthesis, metabolism, detoxification and digestion.

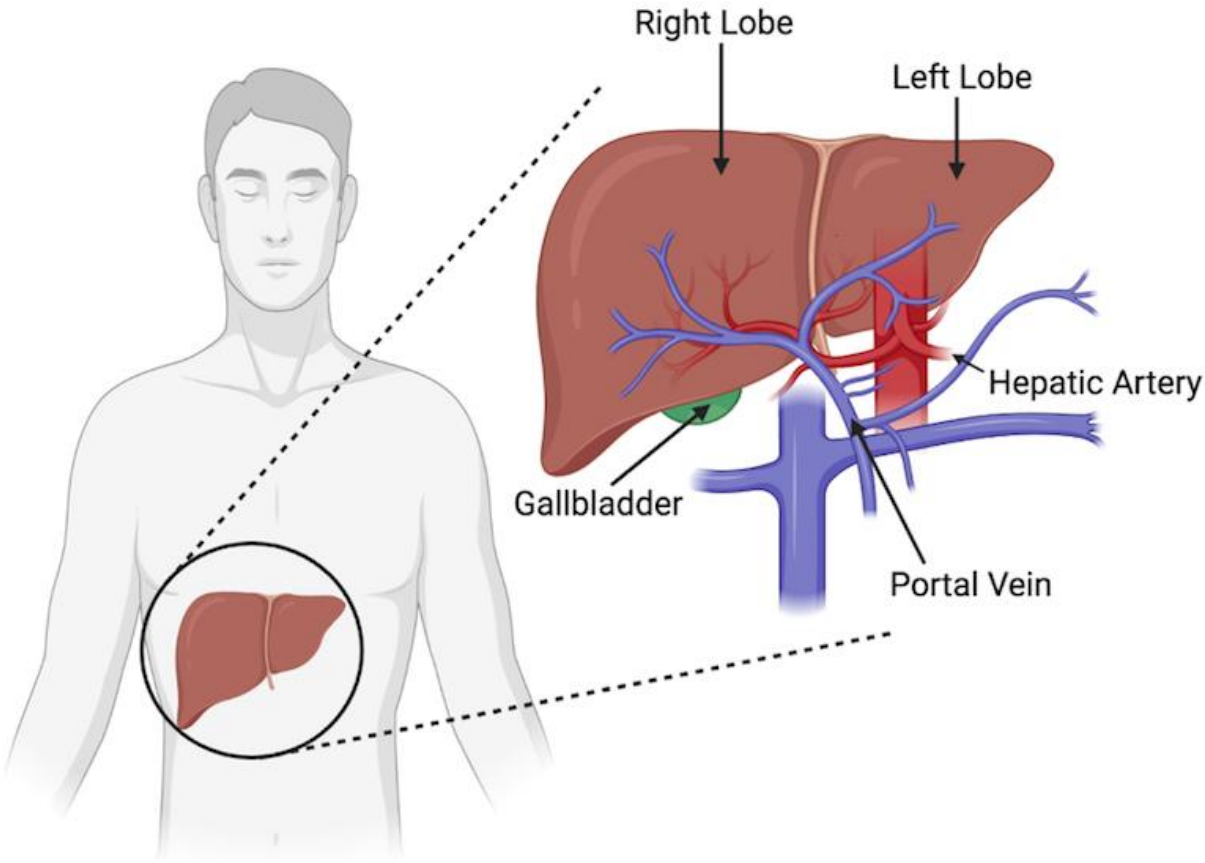

Figure 2 Anatomy of the liver

Figure created in BioRender.com.

Metabolism

The liver is responsible for the levels of circulating proteins present in the blood by regulating both protein synthesis and transamination. Major proteins include albumin, globulin, transport and carrier proteins, fibrinogen and coagulation factors, and proteins of the immune system. The liver is also responsible for glucose homeostasis in order to maintain blood glucose levels through the interplay of glycogenolysis and gluconeogenesis, as well as the synthesis of low and high-density lipoproteins (Feather et al., 2021).

Digestion

Bile is produced in the liver and stored in the gallbladder. Once released from the gallbladder, it enters the small intestine where it helps digest fats.

Immunological

The portal circulation is responsible for the passage of nutrient-rich blood from the gastrointestinal (GI) tract directly to the liver via the portal vein. This circulation of blood enables the liver to remove harmful substances which may have been taken up with the food, prior to entering the systemic circulation. Therefore, the liver has to withstand continuous volumes of dietary, commensal and potentially harmful products. The hepatic immune system is key for the rapid eradication of harmful toxins and molecular waste (Feather et al., 2021). To do so, the liver is comprised of hepatocytes responsible for the synthesis of innate immune proteins including pattern recognition receptors (PRRs) and components of the complement system. The liver is also home to resident macrophages, named Kupffer cells, and is enriched with other innate cells including NK cells, NKT and MAIT cells. (Feather et al., 2021).

Detoxification

The liver is the principal site of detoxification. It is responsible for the catabolism of endogenous hormones such as insulin and oestrogen. The liver is also important for drug and alcohol metabolism, converting exogenous substances into water-soluble forms for excretion via urine (Feather et al., 2021).

1.5 Liver Disease

Liver disease is a major public health concern contributing to 3.5% of deaths globally, with incidence and prevalence of the disease increasing (Asrani, Devarbhavi, Eaton and Kamath, 2019). The most common forms of liver disease include non-alcoholic steatohepatitis (NASH), alcoholic liver disease, chronic hepatitis B and C viral infections and autoimmune diseases. Risk factors include excessive alcohol consumption (ALD), obesity, hypertension and type 2 diabetes (NASH). Europe has the largest consumption of alcohol per capita accompanied by increased rates of obesity (Pimpin et al., 2018) (Williams, 2015). Therefore, it is not surprising

that liver disease is a large burden in Europe and is the third-leading cause of premature death in the UK (Statistics - British Liver Trust, 2019).

Injury of the liver, regardless of aetiology, triggers an inflammatory response through a common pathway. This inflammation drives fibrosis, portal hypertension and ultimately, an increased risk of hepatocellular carcinoma; the third leading cause of cancer-related deaths worldwide (El-Serag, 2012).

Liver cirrhosis is the final common pathway of the disease. It is characterised by extensive scarring of the liver parenchyma, known as fibrosis, and results in impaired function and gross distortion of the liver architecture. The disease typically exists as a long period of asymptomatic inflammation and therefore can remain undiagnosed. As a consequence, approximately 75% of patients are diagnosed with late-stage liver disease. (Pimpin et al., 2018). This is referred to as compensated cirrhosis, whereby lung function remains normal and stable. Cirrhosis can progress to a decompensated phase; patients experience symptomatic manifestations of the disease. Morbidity and mortality of cirrhosis significantly increases following decompensation, with the median survival dropping from 9-12 years to as little as 2 years (D'Amico et al., 2006). This demonstrates the drastic increase in mortality among patients with decompensated cirrhosis. Therefore, late diagnosis of liver disease is a major clinical concern.

The liver function in cirrhosis is commonly classified using the Child-Pugh (CP) scoring system, designed to predict mortality of cirrhotic patients (Tsoris and Marlar, 2022). Patients are grouped into three categories: A (5-6 points), B (7-9 points) and C (10-15 points), which range from good hepatic function to moderate and advanced hepatic dysfunction, respectively. The grading is based on five clinical and laboratory parameters: serum bilirubin, albumin and prothrombin time reflecting synthetic function, ascites, and encephalopathy (neurological disorder) reflecting portal hypertension. Each criterion is sub-grouped based on increasing severity and given a value between 1-3, in order to calculate a CP score between 5 and 15. Following abdominal surgery, CP class A patients have a mortality rate of 10%, CP class B patients have a mortality rate of 30% and patients with CP class C have a 70-80% chance of mortality (Mansour et al.,1997,Garrison et al., 1984).

1.6 The Manifestations of Cirrhosis

Portal hypertension

Portal hypertension represents a major complication of cirrhosis and is of clinical concern when the hepatic venous pressure gradient exceeds 10 mmHg (Garcia-Tsao and Bosch, 2015). It is caused by structural changes in the microcirculation, interference of portal blood flow and increased intrahepatic blood pressure (Figure 3) (Iwakiri, 2014). Cirrhosis results in the dysfunction of liver sinusoidal endothelial cells (LSECS), which act as a frontline defence against liver injury (Iwakiri and Groszmann, 2007). Under physiological conditions, LSECs have roles in blood clearance, hepatocyte growth and angiogenesis (Iwakiri and Groszmann, 2007). However, in cirrhosis, dysfunction of these cells leads to fibrosis, inflammation and portal hypertension as a consequence (Iwakiri, 2014). Furthermore, the production and bioavailability of nitric oxide, a potent vasodilator, is significantly decreased, accompanied by an increase in vasoconstrictors such as thromboxane (Iwakiri and Groszmann, 2007). This further contributes to increased intrahepatic blood pressure. Additionally, hepatic stellate cells become activated and differentiate into myofibroblasts. Myofibroblasts are recruited around the sinusoidal blood vessels, which contributes to increased vascular resistance. Myofibroblasts, in addition to LSECs, produce angiogenic factors such as angiopoietin and vascular endothelial growth factor (VEGF) in order to mediate angiogenesis (formation of new blood vessels) (Iwakiri, 2014). This leads to irregular blood flow and further increases resistance.

In advanced cirrhosis, portal hypertension drives splanchnic vasodilation and activates compensatory mechanisms including the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS, Pedersen, Bendtsen and Møller, 2015). These mechanisms lead to sodium and water retention in order to increase blood volume and cardiac output and maintain systemic blood pressure. However, cirrhotic patients may have underlying cardiomyopathy with a greater risk of cardiovascular collapse, making them unable to further increase cardiac output. As a consequence, renal dysfunction and failure is a common complication in liver cirrhosis (Bucsics and Krones, 2017).

Portal hypertension further exacerbates the disease, leading to other complications including varices, variceal bleeding, hepatic encephalopathy (HE), ascites and bacterial infections.

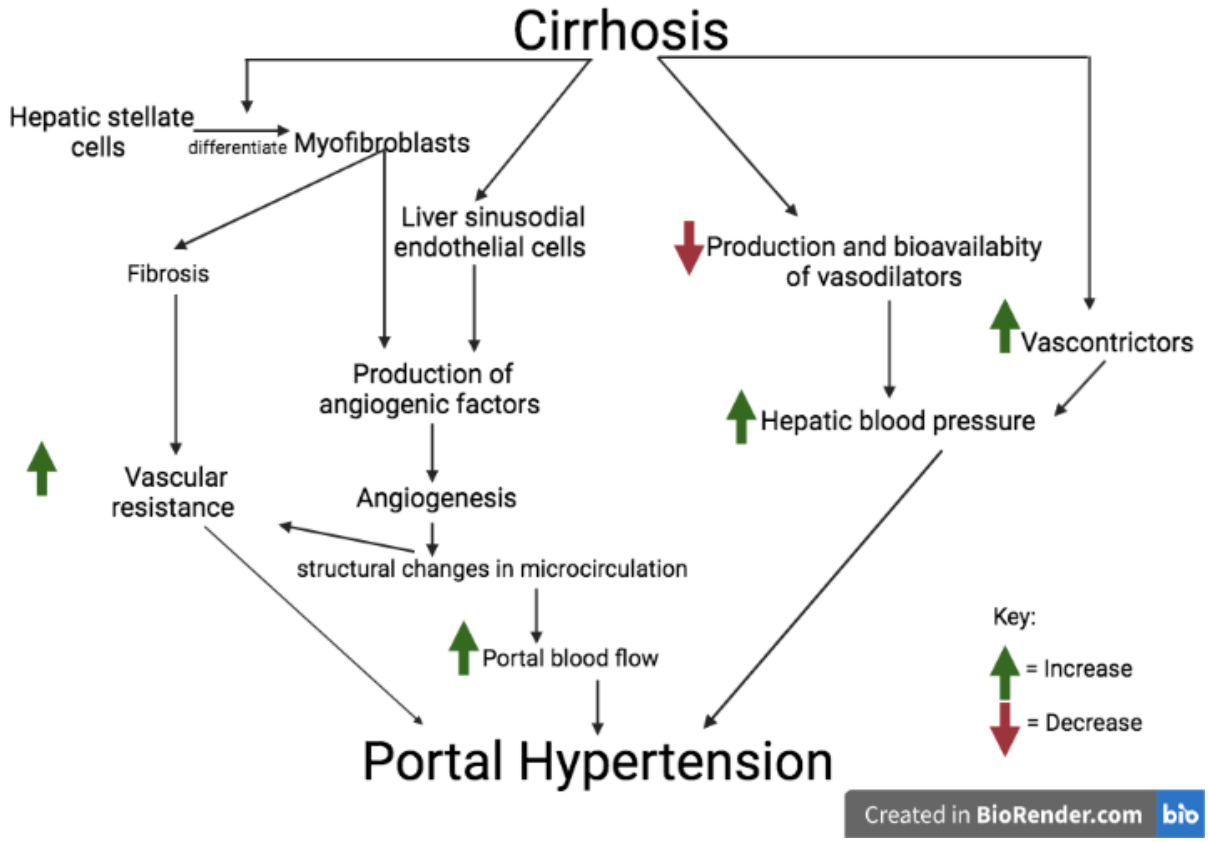

Figure 3 The development of portal hypertension following liver cirrhosis

Figure created in Biorender.com.

Varices and variceal haemorrhage

A direct consequence of portal hypertension is the development of varices (enlarged veins) and variceal haemorrhage. The increased portal pressure results in the development of collateral circulation allowing portal blood to enter the systemic circulation. This can lead to shunts occurring throughout the body including gastroesophageal veins. In a study of 496 patients, 189 patients were diagnosed with varices during the follow-up at 10 (38%) and 20 years (45%) (D'Amico et al., 2014). This demonstrates the progressive onset of varices over time following cirrhosis, with the risk of variceal rupture and bleeding. Mortality due to acute variceal bleeding is approximately 15-20% during the first episode, and as a result patient with advanced cirrhosis (CP-C) have a much higher risk of death (Carbonell et al., 2004).

Encephalopathy

HE can occur as a complication following established cirrhosis. It is a brain dysfunction caused by liver insufficiency and portosystemic shunting in advanced liver disease (Vilstrup et al., 2014). HE exhibits neurological and psychiatric effects including changes in attention, memory, behaviour and personality, irritability, disorientation and alterations of consciousness and motor function (Vilstrup et al., 2014). The initial development of symptomatic HE occurs in 5 to 25% of cirrhotic patients 5 years after diagnosis (Vilstrup et al., 2014), and is most common in elderly cirrhotic patients and patients with non-alcoholic fatty liver disease (NAFLD, Tapper et al., 2019).

Ascites

A common manifestation secondary to liver cirrhosis is ascites, the accumulation of fluid within the peritoneum, which occurs in approximately 50% of patients within 10 years following diagnosis (Cárdenas and Ginès, 2005). Ascites occurs secondary to portal hypertension. Splanchnic vasodilation and the activation of the SNS and RAAS leads to the passage of fluid into the peritoneal cavity (Pedersen, Bendtsen and Møller, 2015). Ascites carries a poor prognosis with a mortality of up to 44% within 5 years from diagnosis (Planas et al., 2006).

Bacterial infections

A further complication of ascites is the development of bacterial infections. Reduced synthetic function of the liver ultimately results in immunosuppression. The intestinal barrier is compromised in advanced liver disease due to increased permeability, gut motility and reduction in the immunological ability of the gut (Feather, Randall, Waterhouse and Kumar, 2020). This leads to bacterial translocation across the gut, particularly *E. coli, Klebsiella pneumoniae* and *Enterococcus* species (Feather, Randall, Waterhouse and Kumar, 2020). Therefore, cirrhotic patients have an increased susceptibility to infection. Approximately one fifth of patients with ascites suffer from spontaneous bacterial peritonitis (SBP) (Feather, Randall, Waterhouse and Kumar, 2020). SBP is associated with poor prognosis, with approximately one third of patients succumbing to renal failure (Karagozian, Rutherford, Christopher and Brown J, 2016). This further illustrates the increased morbidity and mortality of patients with decompensated cirrhosis.

Early diagnosis of bacterial infections is therefore crucial for disease management; however, this is proven difficult. Bacterial infections typically result in non-specific manifestations, which can also reflect systemic inflammation and liver deteriorations. Despite clinical signs of infection, ascites can remain culture negative. Therefore, SBP is diagnosed by an ascitic fluid neutrophil count greater than 250/ml. C-reactive protein (CRP) is shown to be a useful marker to predict bacterial infections in cirrhotic patients without overt infection. However, patients with cirrhosis may present reduced CRP in response to infection. CRP is synthesised primarily by liver hepatocytes. Damage to residential immune cells caused by liver fibrosis may result in depleted synthesis of CRP. Additionally, CRP is a generic biomarker of inflammation and lacks specificity to bacterial infections. This signifies the need for advancements in the accurate and reliable identification of bacterial infections present.

1.7 Role of immune system in pathogenesis and cirrhosis: systemic inflammation and immune dysfunction

The immune system is implicated in both systemic inflammation, which drives fibrosis and cirrhosis, and immune dysfunction in advanced liver disease.

Systemic inflammation

Initial injury to the liver triggers an immune response, which ultimately results in fibrosis and liver cirrhosis. Immune cells become activated by signals released from dead or damaged hepatocytes, termed damage-associated molecule patterns (DAMPs, Koyama and Brenner, 2017). Additionally, signalling mechanisms including NF-κB mediate the activation of immune cells such as Kupffer cells through expression of cytokines and chemokines (Koyama and Brenner, 2017). Kupffer cells are liver-resident macrophages within the sinusoidal capillary, which secrete cytokines including TNF-α, IL-1β, IL-6 and CXCL8 (IL-8) upon activation (Koyama and Brenner, 2017). Following this, macrophages and neutrophils derived from the bone marrow are recruited to the site of injury. M1 macrophages express proinflammatory cytokines such as TNF α, IL-6 and IL-1β which are involved in liver inflammation, whereas M2 macrophages secrete anti-inflammatory molecules e.g. IL-10, TGF-β, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) to promote wound healing (Wang and Ju,

2017) (Mosser and Edwards, 2010). Both Kupffer cells and infiltrating macrophages also secrete TNF-β which is implicated in liver fibrogenesis (Koyama and Brenner, 2017).

Under normal physiological conditions, liver DCs exhibit tolerogenic functions through inducing regulatory T cells (Tregs), which are anti-inflammatory mediators that suppress the immune response by inhibiting T cell proliferation and cytokine production (Zheng and Tian, 2019). However, following injury to the liver, DCs can stimulate hepatic stellate cells, NK cells and T cells, resulting in inflammation (Tokita et al., 2008). DCs activate Th17 cells which produce IL-17 and stimulate Kupffer cells and macrophages to express proinflammatory mediators as well as TGF-β1, a profibrotic cytokine (Koyama and Brenner, 2017). IL-17 also activates hepatic stellate cells to produce collagen type 1 and differentiate into fibrogenic myofibroblasts (Koyama and Brenner, 2017) (Meng et al., 2012).

Liver fibrosis is therefore mediated through the influx and activation of immune cells and acute inflammation which results in the subsequent activation of hepatic stellate cells. Hepatic stellate cells differentiate into myofibroblasts and are responsible for the synthesis of collagen-rich extracellular matrix (ECM), which forms scar tissue in aims to repair the liver parenchyma (Koyama and Brenner, 2017). Prolonged inflammation results in the synthesis of ECM and subsequent cirrhosis. This can progress to impair liver function and impinge on liver architecture.

Immune dysfunction

As cirrhosis progresses, defects arise in both the innate and adaptive immune system resulting in acquired immunodeficiency. This is due to the persistent stimulation of immune cells accompanied with enhanced serum levels of proinflammatory cytokines (Albillos, Lario and Álvarez-Mon, 2014).

Locally, the immune surveillance ability of the liver is diminished due to sinusoidal fibrosis and damage to hepatocytes and resident macrophages (Albillos, Lario and Álvarez-Mon, 2014). Additionally, the clearance of bacteria and bacterial products in the blood is impaired resulting in bacteraemia and chronic immune stimulation. Cirrhosis also compromises the

synthesis and function of immune cells and proteins, impairing the microbicidal ability of these cells.

Furthermore, the effects of cirrhosis extend systemically, compromising the overall protective capability of the immune system. Firstly, neutrophils demonstrate reduced phagocytosis of opsonised bacteria as well as impaired chemotaxis due to reduced adhesion and transendothelial migration into the site of infection (Ono et al., 2004). Furthermore, lymphopenia is common in both B and T cell subsets during cirrhosis (Albillos, Lario and Álvarez-Mon, 2014). This occurs along with memory cell dysfunction and diminished proliferation of circulating lymphocytes (Albillos, Lario and Álvarez-Mon, 2014). This highlights the shift between a proinflammatory state in stable cirrhosis to immunodeficiency in severely decompensated liver disease and acute-on chronic liver failure (ACLF).

Additionally, as cirrhosis progresses, activated immune cells and proinflammatory cytokines accumulate within the gut wall (Albillos, Lario and Álvarez-Mon, 2014). This intestinal inflammation disrupts the gut wall barrier, affecting the tight epithelial junctions, increasing the permeability to bacteria and thus increased bacterial translocation across the gut reaching the systemic circulation resulting in severe infections.

The altered host response results in inappropriate inflammatory responses and immune dysfunction, ultimately predisposing cirrhotic patients to infection. This impaired response to bacterial challenge and increased susceptibility to infection are synergistically responsible for multi-organ failure and mortality in liver disease.

1.8 Acute-On Chronic Liver Failure

ACLF is increasingly recognised as a new physiologically distinct entity of liver disease. It is characterized by acute deterioration of liver function and is often associated with a hepatic or extrahepatic precipitating event and end-organ failure (Arroyo, Moreau and Jalan, 2020). The precipitating event which exacerbates liver disease and leads to ACLF is not always identified. However, in most cases, the syndrome is triggered by trauma, surgery, variceal bleeding and infection. Subsequently, ACLF has a high risk of short-term mortality due to the

failure of one or more organs, carrying a 28-day mortality of 32% (Moreau et al., 2013). The degree of end-organ failure determines the outcome, with approximately 50-90% of ACLF patients succumbing to short-term mortality due to multi-organ failure (Jalan and Williams, 2002).

The precise pathophysiology of ACLF is unclear, however it is believed to be a result of the unregulated inflammation and altered host response as previously mentioned. Systemic inflammation is largely implicated in the pathophysiology and is associated with severe encephalopathy, infection and renal failure, with patients presenting elevated levels of pro- and anti-inflammatory mediators including TNF-α, IL-2, IL-6, IL-10 and IFN-γ (Gustot, 2011). Furthermore, infection is a common occurrence in ACLF, which further aggravates the proinflammatory response and is associated with significant morbidity and mortality (Arroyo, Moreau and Jalan, 2020). Therefore, the severity of the inflammation and occurrence of new bacterial infections results in dismal outcomes in patients with ACLF. This emphasises the severity of developing ACLF and demands for a better understanding of the pathophysiology in order to identify novel biomarkers and develop therapeutic strategies to combat this condition.

The CANONIC prospective study demonstrated variability of ACLF whereby approximately 50% of cases recovered from organ dysfunction (Gustot et al., 2015). This suggests that ACLF has numerous stages, with the early stage, also termed grade 1, being potentially reversible in a subset of patients. Therefore, rapid diagnosis of ACLF and early intervention to treat the precipitating events may be pivotal to improve outcomes of these patients.

1.9 Current Strategies for the Treatment of Liver Disease

Lifestyle changes are advised to slow down or reduce disease progression. Such changes may include abstinence from alcohol, weight loss, regular exercise and vaccinations.

If patients experience complications associated with cirrhosis, medications are available to manage symptoms. Beta blockers are prescribed in order to reduce the risk of variceal bleeding as a consequence of portal hypertension. Additionally, diuretics can reduce the

accumulation of ascites. Furthermore, in the case of bacterial infections, broad spectrum antibiotics are prescribed as standard treatment.

Liver transplantation is the only treatment option in cases of advanced liver disease where the liver is severely damaged. However, the availability and suitability of a donor liver is a major limitation.

Albumin infusion therapy remains controversial for the management of cirrhosis and ascites drainage. Albumin is a protein produced in the liver which functions as a transport protein for calcium, zinc, bilirubin, hormones and drugs. It also maintains oncotic pressure within the plasma to prevent fluid leaking out into blood vessels. Patients with liver disease experience a reduced production of albumin, due to liver damage.

In a randomised study, Sort and colleagues demonstrated that the administration of intravenous albumin reduced mortality in cirrhotic patients with SBP by preventing renal impairment and failure (Sort et al., 1999). In agreement with this, another study compared the efficacy of albumin therapy with hydroxyethyl starch and found that that albumin was superior in preventing renal failure in SBP patients (Fernández et al., 2005). The precise mechanisms remain unclear; however, it is believed to be an interplay of oncotic effects, improved cardiac function and a reduction in arterial vasodilation in order to regulate blood pressure. Despite this success, albumin infusion is inefficient in reducing renal dysfunction and survival in decompensated patients with non-SBP infections. In a study by Thévenot et al., 2015, 8.3% of participants developed pulmonary oedema after albumin administration and two patients died as a result. This suggests that albumin administration may increase the risk of pulmonary oedema in decompensated cirrhosis in the presence of infections other than SBP. Furthermore, a recent systemic review concluded that albumin infusion did not have a clinical benefit against non-SBP infections but suggested that more evidence is required on its efficacy in regard to ACLF (Wong et al., 2020). Therefore, there is a need to stratify subgroups of patients in order to improve clinical outcomes.

Taken together, the different clinical manifestations of cirrhosis include stable compensated cirrhosis, varices, variceal bleeding, ascites, bacterial infections, hepatic encephalopathy, and

hepatocellular carcinoma. The immune system plays a pivotal role in cirrhosis, with systemic inflammation progressing into a state immune dysfunction during advanced liver disease. It is likely that immune profiles and deficiencies related to cirrhosis may differ in accordance with disease state. It can thus be speculated that differences in soluble and cellular levels and function may correlate with disease severity, treatment response and outcome. These distinct differences will allow us to further understand immune responses in liver disease and suggest improved ways for diagnosis and intervention.

2. Hypothesis, Research Question and Objectives

Hypothesis

Stratification of cirrhotic patients by immune characteristics in the peripheral blood can predict infection and outcome.

Aim

To gain further understanding of immune characteristics in peripheral blood and ascitic fluid of cirrhotic patients and determine its' significance in disease severity and outcome.

Objectives

- Analyse cytokine, chemokine, immune cell phenotype and functional marker profiles in peripheral blood and ascites

- Integrate cellular, soluble and clinical data to identify possible correlations of immune signatures with clinical outcomes

3. Materials and Methods

3.1 Participants

Individuals attending the hepatology ward as day case patients and those admitted to University Hospital of Wales Cardiff between December 2018 and December 2021 were invited to take part in the study. Individuals with clinically diagnosed cirrhosis aged 18 years or over were eligible for participation. Patients with severe immune deficiencies for example diagnosis of AIDS, anti-rejection transplant drugs or high dose corticosteroids were, however, exempt. Informed consent was obtained in keeping with research ethics approval for this project (Immune fingerprinting of bacterial infections in decompensated cirrhosis IRAS project ID 235693). Participants donated a peripheral blood sample with a paired ascites sample if large volume ascites was present, these samples were obtained by appropriately trained NHS staff. Healthy donors were recruited through the 'Coordinating COVID-19 asymptomatic testing programmes in university settings: providing insight on acquired immunity across the student population' study, funded by the UKRI COVID-19 National Core Study Immunity programme. The study received ethical approval from Cardiff University School of Medicine Research Ethics Committee (SREC reference: SMREC 21/01).

3.2 Sample Preparation

Heparinised blood samples were layered over Lymphoprep (Alere International Limited) in a 50 ml Falcon tube and centrifuged along with heparinised ascitic fluid at 2000 rpm for 20 minutes at room temperature. Cells present in the blood form a hazy interface which was aspirated using a Pasteur pipette into a fresh Falcon tube and R+ (RPMI-1640, penicillin, streptomycin, L-glutamine and Fetal Calf Serum) (Gibico and PAA) was added to make up 40 ml volume. Ascitic fluid above the pellet was transferred into cryotubes and frozen. The pellet was resuspended with R+ to make up 40 ml and centrifuged with the plasma cells at 2000 rpm for 10 minutes at room temperature. Plasma, forming a layer above the Lymphoprep, was then transferred into cryotubes and frozen. Following centrifugation, the supernatant was disposed of, and the pellet was released and resuspended in 20 ml R+ and centrifuged at 1600 rpm for 5 minutes at room temperature. The supernatant was poured away and the

pellet was resuspended in 5 ml red blood cell (RBC) lysis buffer (Biolegend) and incubated for 3 minutes at room temperature. 20 ml of R+ was added to inactivate the lysis buffer and was then centrifuged at 1600 rpm for 5 minutes at room temperature. The supernatant was disposed of, and the pellet was resuspended in 5 ml of PBS (Gibco). 50 µl of this suspension and 0.5 µl of propidium iodide (Sigma-Aldrich) was pipetted into a 5 ml FACS tube and agitated before it was loaded onto a 24-tube rack and placed into the Novocyte AutoSampler. A cell count was run at medium speed. The remaining suspension was centrifuged at 16000 rpm for 5 minutes at room temperature. The supernatant was poured away, the pellet was released and resuspended in PBS at a cell number of $5x10^6$ PBMC/ml.

Live/Dead staining

50 µl of prepared cells were added to wells on a 96-well plate for 3 conditions, T cells, non-conventional T cells (NCT) and myeloid-derived suppressor cells (MDSC), and fluorescence minus one (FMO) controls. Invitrogen fixable live/dead-reconstituted in 50 µl DMSO and diluted 1:10 with PBS before 3 µl was added to each well.

Cell surface staining

Antibodies were added to the appropriate wells as listed in Tables 1-3.

Table 1 T Cell Antibody Panel

Antibody	Supplier	Code	Clone	Final Dilution	Volume Antibody added
CD3-FITC	BioLegend	300406	UCHT1	1:50	1µl
CD56-BV421	BioLegend	300554	HCD56	1:50	1µl
CD4-BV605	BioLegend	344710	RPA-T4	1:50	1µl
CD8-APC-H7	BD BioScience	560179	SK1	1:50	1µl
CD14-BV500	BD BioScience	561391	M5E2	1:50	1µl
CD19-BV500	BioLegend	309822	4B4-1	1:50	1µl
CD69-PECy7	BioLegend	310912	FN50	1:50	1µl
PD1-APC	BioLegend	367405	NAT105	1:50	1µl
Ki67-PE	BioLegend	350504	Ki67	1:25	2 µl

Table 2 Non-Conventional T Cell (NCT) Antibody Panel

Antibody	Supplier	Code	Clone	Final Dilution	Volume Antibody added
CD3-FITC	BioLegend	300406	UCHT1	1:50	1μl
CD8-APC-H7	BD BioSciences	560179	SK1	1:50	1μl
CD14-BV500	BD BioSciences	561391	M5E2	1:50	1μl
CD19-BV500	BioLegend	309822	4B4-1	1:50	1μl
Va7.2-BV605	BioLegend	351720	3C10	1:16.7	3μl
CD161-APC	Miltenyi Biotec	130-092-678	191B8	1:25	2μl
TCR G/D PE Dazzle	BioLegend	331226	B1	1:16.7	3μl
Ki67-PE	BioLegend	350504	Ki67	1:25	2 μl

Table 3 Myeloid-Derived Suppressor Cell (MDSC) Antibody Panel

Antibody	Supplier	Code	Clone	Final Dilution	Volume Antibody added
CD3-BV421	BioLegend	300434	UCHT1	1:100	0.5μl
CD19-BV421	BioLegend	302234	HIB19	1:50	1μl
CD56-BV421	BioLegend	318328	HCD56	1:100	0.5μl
CD66b-APC	BioLegend	305117	G10F5	1:100	0.5μl
CD15-BV785	BioLegend	323044	W6D3	1:100	0.5μl
CD14-APC-H7	BD BioSciences	560180	MΦP9	1:50	1μl
CD11b-FITC	BioLegend	301329	ICRF44	1:100	0.5μl
CD33-BV605	BioLegend	366612	P67.6	1:50	1μl
CD40-PE	Beckman Coulter	IM1936U	MAB89	1:25	2μl
HLA-DR-PECy7	BioLegend	307616	L243	1:25	2μl

Fluorescence Minus One and Compensation Controls

Fluorescence Minus One (FMO) controls for Ki67 PE, PD1 APC, CD69 PE-Cy7, HLA-DR, CD33, CD40 and CD11b were run on each plate. FMO controls determined the upper boundary of background signal of the fluorescent label, allowing the identification of positive populations in the multicolour assay. In order to correct for fluorescence spill over, compensation was run on BV421, BV605, BV785, FITC, PE, PerCP, PE-Cy7, APC, APC-H7 for each run.

The plate was placed in the fridge for 15 minutes before it was washed twice with FACS buffer, centrifugated at 1400 rpm for 3 minutes and resuspended in 120 μl FACS buffer. One drop of ARC negative beads was then added into the aqua live/dead compensation well. MDSC antibody panel were ready to be run on the Novocyte flow cytometer.

Fix: Perm concentrate (1:4, Thermo Fisher) was added to the T-cell and NCT panel and placed in a dark area for 45 minutes before it was spun down. The panels were then washed in 1:10 perm buffer before 2% rat serum perm buffer (JBios) was added to each relevant well and placed in the fridge for 15 minutes. Ki67-PE antibody was then added to the T-cell and NCT panel.

The plate was then refrigerated for 15 minutes before being washed twice with perm buffer, centrifugated at 1400 rpm for 3 minutes, resuspended in 120 μl perm buffer and run on the Novocyte flow cytometer.

3.3 Magnetic Luminex Assays

In order to measure concentrations of soluble analytes in patient samples, R&D Systems magnetic Luminex assays (4-plex) and magnetic Luminex performance assays (29-plex) were conducted. Unlike traditional ELISA, Luminex enables multiple cytokines to be measured simultaneously compared to a single analyte per assay. The 33 analytes measured are listed in Table 4. Plasma and ascites samples were thawed and centrifuged at 16,000 x *g* for 4 minutes. Reagents and standards were prepared according to the manufacturer's instructions

for a 96-well microplate plate was used. 50 µl per well of sample, standard or control were added to each plate. The dilute microparticle cocktail was resuspended by vortexing prior to adding 50 µl to each well in as little light as possible. The microplates were secured with the foil plate sealers on the shaker at 500 rpm, in the cold room, overnight. To ensure the microparticles remain at the bottom of the microplate, a magnetic device designed to accommodate the 96-well microplate. The plate was secured on the magnetic device for 1 minute prior to removing the liquid. The wells were washed with wash buffer and placed on the shaker at 800 ± 50 rpm for 1 minute before securing onto the magnetic device for 1 minute prior to removing the liquid again. This procedure was repeated for 3 washes. 50 µL of the diluted biotin-antibody cocktail was added to each well, and the microplates were secured with foil plate sealers and incubated at room temperature on the shaker at 800 ± 50 rpm. The microplates were washed with wash buffer 3 times as previously described. The microplate was incubated for 30 minutes, at room temperature on the shaker at 800 ± 50 rpm, following the addition of 50 µl of streptavidin-PE was added to each well. The microplates were washed with wash buffer 3 times as previously described. The microparticles were resuspended in 100 µl of wash buffer and incubated for 2 minutes on the shaker at 800 ± 50 rpm prior to running the plates on the Luminex machine.

Table 4 List of Analytes Measured in 4-Plex and 29-Plex Magnetic Luminex Assays

Analyte	Magnetic Luminex Assay
Fas Ligand	4-Plex
ICAM/ CD54	4-Plex
IL-12/ IL-23 p40	4-Plex
TNF-β	4-Plex
PD-L1	29-Plex
CCL2/ MCP-1	29-Plex
CCL3/ MIP-1a	29-Plex
CCL4/ MIP-1b	29-Plex
CCL5/ RANTES	29-Plex
CD40L	29-Plex
CXCL10/ IP-10	29-Plex
G-CSF	29-Plex
GM-CSF	29-Plex
IFN-α	29-Plex
IFN-γ	29-Plex
IL-12 p70	29-Plex
IL-17α	29-Plex
IL-1α	29-Plex
IL-1β	29-Plex
IL-4	29-Plex
IL-6	29-Plex
IL-10	29-Plex
IL-2	29-Plex
IL-5	29-Plex
IL-7	29-Plex
IL-8/ CXCL8	29-Plex
PDGF-AA	29-Plex
PDGF-AB/BB	29-Plex

3.4 Statistical Analysis

NHS laboratory parameters were analysed using Kruskall-Wallis and Dunn's multiple comparison tests. Standard curves were generated in GraphPad Prism (version 8.4.3) using the standard concentrations provided by each kit and MFI values produced by Luminex. From this, concentrations of each analyte were interpolated, and Kruskall-Wallis and Dunn's multiple comparison tests were performed on plasma samples, and Wilcoxon pairwise

analyses were performed on paired plasma and ascites samples. For flow cytometry, statistical comparisons were made using Kruskall-Wallis and Dunn's multiple comparison tests for peripheral blood alone and using Wilcoxon matched pairs signed rank tests for paired peripheral samples. Corrections for multiple comparisons were performed using Dunn's tests as recommended by GraphPad Prism version 9. Reported p-values were multiplicity adjusted p-values for each comparison, using a family-wise significance and confidence level of 0.05. This was an exploratory pilot study and therefore p-values marginally above the 0.05 limit were reported as trends observed may have potential interest within a larger cohort in future work.

4. Clinical and Flow Cytometry Analysis

4.1 Patient Demographics

A total of 115 patients were recruited following acute admission to the University Hospital of Wales or following day case ward review (Table 5). In this cohort of liver disease patients, there was a male predominance (67%) with a median age of 61. The predominant aetiologies were alcoholic liver disease (ALD; 68.7%) and non-alcoholic steatohepatitis (NASH; 20%). Although NASH is the most common cause of chronic liver disease in Wales, ALD-related admissions remain leading cause of cirrhosis and hospital admission and this is reflected in this cohort (Wales Liver Registry, T Pembroke personal communication). These were followed by ALD and concurrent hepatitis C virus (HCV) infection (5.2%), cryptogenic liver disease (3.5%), autoimmune hepatitis (AIH) (1.7%) and cardiac cirrhosis (0.9%). The complexity and diversity of liver cirrhosis is demonstrated by the different causes of admissions. Participants were predominantly admitted with complications of ascites (17.4%), variceal bleeding (7%), and infections which were not SBP (pneumonia, urinary tract, osteomyelitis and gastroenteritis 13%) and SBP (3.5%). Other causes of admission were alcoholic hepatitis (9.6%), HE (7%), fall/fit (5.2%), non-variceal upper gastrointestinal (UGI) bleeding (4.3%), lymphoma (0.9%) and jaundice/synthetic failure without clear precipitant (0.9%). One patient was admitted with abdominal pain and was subsequently diagnosed with lymphoma; this individual was excluded from this study due to the potential impact of lymphoma on the immune response. Individuals with stable cirrhosis with and without chronic decompensation were also recruited; patients with ascites who underwent elective paracentesis (EP:12.2%), and patients with no acute illness (CP-B) (10.4%), and no acute illness (CP-A) (7.8%), respectively. Most patients recruited had a CP score of C (48.7%), followed by CP-B (40%), and 15 patients had a CP-A (11.3%). Taken together, this distribution of pathologies across the patient cohort highlights that most participants had advanced liver disease and hepatic dysfunction.

The median age of patients with liver disease in Wales is 63 and is also male-dominant (Appendix 1, Wales Liver Registry). These similarities mean that we can be confident the study cohort is representative of liver disease in Wales.

The heterogeneity of liver disease makes stratification of patients into clear groups challenging. We aimed to recruit approximately 10 participants per group for adequate statistical analysis. Participants were grouped into 7 groups: healthy donors, no acute illness (CP-A), no acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and ACLF.

Healthy donors

Sixteen healthy donors were recruited through the 'Coordinating COVID-19 asymptomatic testing programmes in university settings: providing insight on acquired immunity across the student population' study, funded by the UKRI COVID-19 National Core Study Immunity programme. The study received ethical approval from Cardiff University School of Medicine Research Ethics Committee (SREC reference: SMREC 21/01). Blood was taken from 7 males and 9 females with ages ranging from 24-62 years.

No acute illness (CP-A)

Five males and 4 females were categorised as no acute illness (CP-A). Patients were between the ages of 50-69 years (median age of 58) and had ALD (44.4%), NASH (33.3%) or HCV infection (22.2%). All patients were CP-A, with relatively normal hepatic function. Patients attended as outpatients primarily for a Critical Flicker Frequency test for assessment of minimal HE (2 had CFT consistent with minimal HE, 6 no minimal HE).

No acute illness (CP-B)

Thirteen patients with CP-B were recruited with no acute illness. 69% were males with a median age of 60 years (age ranged between 47 and 65 years). Patients had ALD (46.2%), NASH (38.5%) or cryptogenic liver disease (7.7%).

Chronic decompensation (no infection)

Twenty-seven patients recruited were categorized with chronic decompensation (CP-B 51.9% or CP-C 48.1%). Of these patients, 19 were male (70.3%) with ages ranging from 42-75 years (median age of 62). 77.8% of patients had ALD, followed by NASH (14.8%), cardiac cirrhosis (3.7%) and cryptogenic liver disease (3.7%). The majority of patients had ascites, 29.6% of patients were admitted with ascites and a further 29.6% attended for elective paracentesis

(Ascites EP), 4 patients were classified as fall/fit (14.8%) and 1 patient was admitted with HE (3.7%).

Acute decompensation (no infection)

Thirty-eight patients were recruited with acute decompensation. 71.1% of acutely decompensated patients were male with a median age of 62 (age ranged from 42-78). The most common aetiology was ALD (63.2%), followed by NASH (23.7%), ALD with concurrent HCV infection (10.5%) and one patient had AIH (2.7%). Patients were admitted with various manifestations including ascites (32.4%), variceal bleeding (18.9%), HE (16.2%), alcoholic hepatitis (13.5%), non-variceal UGI bleeding (5.4%) and non-SBP infection (2.6%). Patients were predominately CP-C (60.5%), 31.6% were CP-B and 3 patients were CP-A (7.9%). Patients with CP-A disease had variceal bleeding which was classed as an acute decompensating event. All individuals had negative culture samples when taken.

Acute decompensation (infection)

Twelve acutely decompensated patients were admitted due to infection. This subgroup was predominately male (75%) with a median age of 58.5 (ages ranged from 48-74). The majority of patients had ALD (75%), with 2 patients having cryptogenic liver disease (16.7%) and 1 with NASH (8.3%). 83.3% of patients were diagnosed with non-SBP infections, including pneumonia, gastroenteritis and urinary tract infection, whereas 2 patients suffered SBP (16.7%). 50% of patients were CP-C, 41.7% of patients were CP-B and 1 patient (8.3%) was CP-A.

ACLF

Sixteen patients were recruited with ACLF. ACLF is a complex but well-defined syndrome defined by liver and other organ failures and is associated with a high mortality. This group contains patients with sterile inflammation or infection. The male-to-female ratio within this group was equal and most patients were in the range of 60-69 years of age (median age of 55.5). All ACLF patients had ALD; one patient had concurrent HCV infection. Patients were admitted primarily with alcoholic hepatitis (37.5%) and infection being non-SBP infections (25%) or SBP (12.5%), followed by variceal bleeding (6.3%), jaundice/synthetic function

(6.25%), non-variceal UGI bleeding (6.3%) and HE (6.3%). As ACLF is associated with high risk of short-term mortality, 87.5% of patients were CP-C signifying advanced hepatic dysfunction.

Table 5 Patient Demographics

		All patients	Healthy Donors	No acute illness (CP-A)	No acute illness (CP-B)	Chronic decomp (no infection)	Acute decomp (no infection)	Acute decomp (infection)	ACLF
Sex	Male	**77** (66.96%)	**7** (43.7%)	**5** (55.6%)	**9** (69.2%)	**19** (70.4%)	**27** (71.05%)	9 (75%)	**8** (50%)
	Female	**38** (33.04%)	**9** (56.3%)	**4** (44.4%)	**4** (30.8%)	**8** (29.6%)	**11** (28.95%)	3 (25%)	**8** (50%)
Age	20-29	---	**4** (25%)	---	---	---	---	---	---
	30-39	**2** (1.7%)	**7** (43.8%)	---	---	---	---	---	**2** (12.5%)
	40-49	**13** (11.3%)	**2** (12.5%)	---	**1** (7.7%)	**4** (14.8%)	**3** (7.89%)	**2** (16.7%)	**3** (18.8%)
	50-59	**36** (31.3%)	**1.** (6.25%)	**5** (55.6%)	**5** (38.5%)	**8** (29.6%)	**9** (23.68%)	**4** (33.3%)	**5** (31.3%)
	60-69	**38** (33.04%)	**2.** (12.5%)	**4** (44.4%)	**2** (15.4%)	**7** (25.9%)	**15** (39.47%)	**4** (33.3%)	**6** (37.5%)
	70-79	**25** (21.74%)	---	---	**4** (30.8)	**8** (29.6%)	**11** (28.95%)	**2** (16.7%)	---
	80+	**1** (0.9%)	---	---	**1** (7.7%)	---		---	---
Aetiology	ALD	**79** (68.7%)		**4** (44.4%)	**6** (46.2%)	**21** (77.8%)	**24** (63.16%)	**9** (75%)	**15** (93.8%)
	NASH	**22** (20%)		**3** (33.3%)	**5** (38.5%)	**4** (14.8%)	**9** (23.68%)	**1** (8.3%)	---
	ALD/ HCV	**7** (5.2%)		2 (22.2%)	---	---	**4** (10.53%)	---	**1** (6.25%)
	Cryptogenic	**4** (3.5%)		---	**1** (7.7%)	**1** (3.7%)	---	**2** (16.7%)	---
	AIH	**2** (1.7%)		---	**1** (7.7%)	---	**1** (2.63%)	---	---
	Cardiac	**1** (0.9%)		---	---	**1** (3.7%)	---	---	---
Cause of admission	Ascites	**20** (17.39%)		---	---	**8** (29.6%)	**13** (34.21%)	---	---
	Variceal bleeding	**8** (6.96%)		---	---	---	**7** (18.42%)	---	**1** (6.25%)
	Non-SBP infection	**15** (13%)		---	---	---	**1** (2.63%)	**10** (83.3%)	**4** (25%)
	Ascites EP	**14** (12.2%)		---	---	**14** (51.85%)	---	---	---
	No acute illness CP-B	**13** (11.3%)		---	**13** (100%)	---	---	---	---
	Alcoholic hepatitis	**11** (9.6%)		---	---	---	**5** (13.16%)	---	**6** (37.5%)
	No acute illness CP-A	**9** (7.8%)		**9** (100%)	---	---	---	---	---
	HE	**8** (7%)		---	---	**1** (3.7%)	**6** (15.79%)	---	**1** (6.25%)
	Fall/fit	**6** (5.2%)		---	---	**4** (14.8%)	**2** (5.26%)	---	---
	Non variceal UGI bleeding	**5** (4.3%)		---	---	---	**4** (10.53%)	---	**1** (6.25%)
	SBP	**4** (3.5%)		---	---	---	---	**2** (16.7%)	**2** (12.5%)
	Lymphoma	**1** (0.9%)		---	---	---	---	---	---
	Jaundice/ synthetic failure	**1** (0.9%)		---	---	---	---	---	**1** (6.25%)
Child-Pugh Score	A	**13** (11.3%)		**9** (100%)		---	**3** (7.89%)	**1** (8.3%)	---
	B	**46** (40%)		---	**13** (100%)	**14** (51.9%)	**12** (31.58%)	**5** (41.7%)	**2** (12.5%)
	C	**56** (48.7%)		---		**13** (48.1%)	**23** (60.53%)	**6** (50%)	**14** (87.5%)
Total		**115**	**16**	**9**	**13**	**27**	**38**	**12**	**16**

4.2 Clinical Parameters

Routine laboratory tests were performed on all patients at the University Hospital of Wales (Figure 4). The median age of patients recruited with ACLF was lower compared to acute decompensation (no infection) patients (p=0.0549, Figure 4A). Haemoglobin levels were reduced in patients with acute decompensation (no infection) and ACLF compared to no acute illness (CP-A) patients (p=0.074 and 0.071, respectively), as shown in Figure B. There was a significant increase in absolute white cell count (WCC) and absolute neutrophil count in ACLF patients compared to no acute illness (CP-A) (p=0.0023 and 0.001, Figures 4C and 4D, respectively) no acute illness (CP-B) (p=0.0156 and 0.0164, respectively). Patients with ACLF also exhibited increased absolute WCC compared with acute decompensation (no infection) patients (p=0.055). Figure 4E shows the absolute monocyte count was significantly elevated in ACLF patients in comparison with no acute illness (CP-A), no acute illness (CP-B) and chronic decompensation (no infection) (p=0.0005, 0.0192 and 0.0208, respectively). No significant difference was observed in the absolute lymphocyte count across patient groups (Figure 4F). However, there was a significant range in in lymphocyte count across every group with a third of patients in each group falling into the lymphogenic range (Figure 4F).

Lymphocytes are highly involved in the orchestration of an immune response; therefore, it is striking to observe no difference in lymphocyte counts between patient groups. Despite this, the total lymphocyte count may mask distinct patterns within different lymphocyte subsets. As anticipated, patients with ACLF presented with distinct clinical characteristics. ACLF occurs as a result of unregulated inflammation and altered host response, and this is reflected by the elevated blood levels of total white blood cells, neutrophils and monocytes. Patients with ACLF were also associated with anaemia and were a younger subgroup.

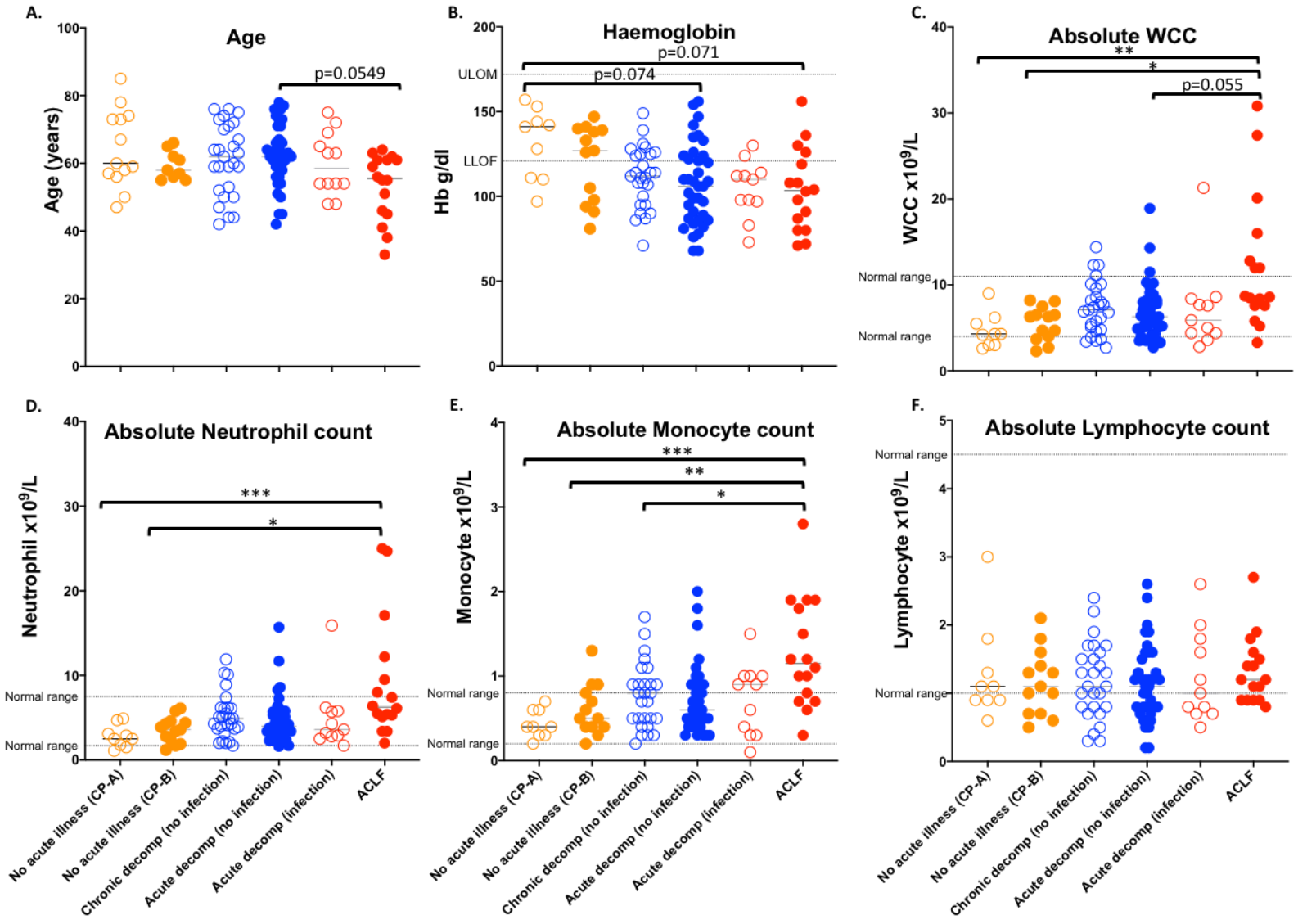
A.
Age
Age (years)
p=0.0549
B.
Haemoglobin
Hb g/dl
p=0.071
p=0.074
ULOM
LLOF
C.
Absolute WCC
WCC x10⁹/L
**
*
p=0.055
Normal range
Normal range
D.
Absolute Neutrophil count
Neutrophil x10⁹/L
***
*
Normal range
Normal range
E.
Absolute Monocyte count
Monocyte x10⁹/L
***
**
*
Normal range
Normal range
F.
Absolute Lymphocyte count
Lymphocyte x10⁹/L
Normal range
Normal range
No acute illness (CP-A)
No acute illness (CP-B)
Chronic decomp (no infection)
Acute decomp (no infection)
Acute decomp (infection)
ACLF

Figure 4 Routine NHS laboratory clinical parameters in 115 patients with liver disease

Comparisons were made against (A) age, (B) haemoglobin, (C) absolute WCC, (D) absolute neutrophil count, (E) absolute lymphocyte count and (F) absolute monocyte count across patient groups: no acute illness (CP-A), no acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Dotted lines represent the normal adult range of clinical parameters. Statistical comparisons were made using Kruskall-Wallis and Dunn's multiple comparison tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and the horizontal line represents the median value.

4.3 Cellular Phenotype and Functional Marker Profiles

To determine immune cell phenotype and functional marker profiles in the peripheral blood of cirrhotic patients, conventional and non-conventional lymphocytes were analysed by multiparameter flow cytometry. In accordance with clinical parameters, patients were stratified into healthy donors, acute illness (CP-A), acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and ACLF.

NK cells

The proportion of NK cells within all live lymphocytes as well as the proportion of CD56 bright NK cells within the NK cell population and the expression of the activation markers CD69, Ki67 and PD1 expression on NK cells was measured. No significant differences were observed between the proportion of NK cells, CD56 bright NK cells and expression of NK functional markers across patient groups (Figure 5).

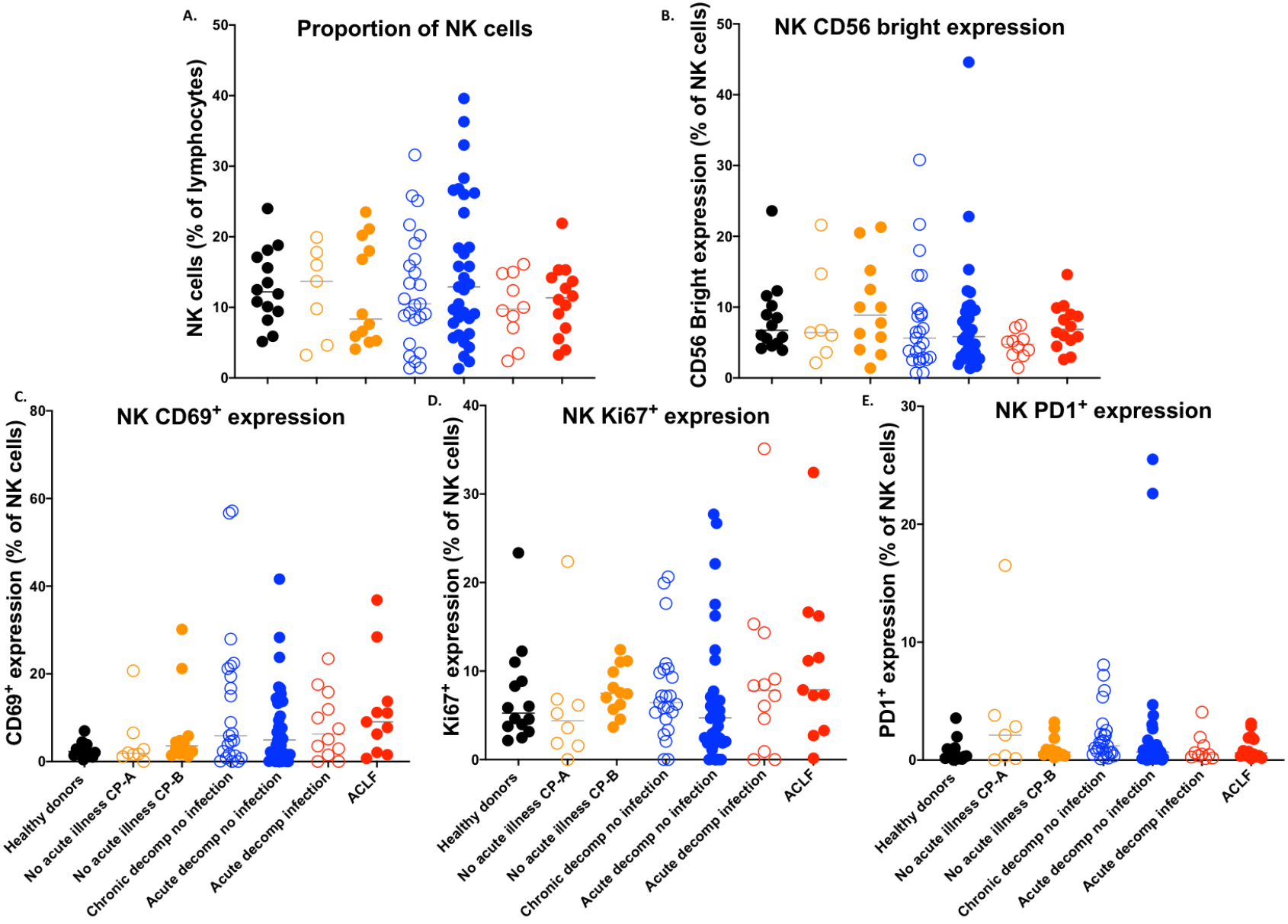
A.
Proportion of NK cells
NK cells (% of lymphocytes)
B.
NK CD56 bright expression
CD56 Bright expression (% of NK cells)
C.
NK CD69+ expression
CD69+ expression (% of NK cells)
D.
NK Ki67+ expresion
Ki67+ expression (% of NK cells)
E.
NK PD1+ expression
PD1+ expression (% of NK cells)
Healthy donors
No acute illness CP-A
No acute illness CP-B
Chronic decomp no infection
Acute decomp no infection
Acute decomp infection
ACLF

Figure 5 Conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 115 liver disease patients and 16 healthy donors

Comparisons were made against (A) the proportion of NK cells within all live lymphocytes, (B) the proportion CD56 bright NK cells within the NK cell population and the expression of the activation markers (C) CD69, (D) Ki67 and (E) PD1 on NK cells across patient groups: no acute illness (CP-A), no acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Kruskall-Wallis and Dunn's multiple comparison tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and the horizontal line represents the median value.

$CD4^+$ T cells

The proportion of T cells which were $CD4^+$ and the markers CD69, Ki67 and PD1 were measured (Figure 6). The proportion of $CD4^+$ T cells was significantly increased in patients with chronic decompensation (no infection) and ACLF patients in comparison with healthy donors (p=0.034 and 0.017, respectively; Figure 6A). No significant differences were observed between functional markers of $CD4^+$ T cells across participant groups.

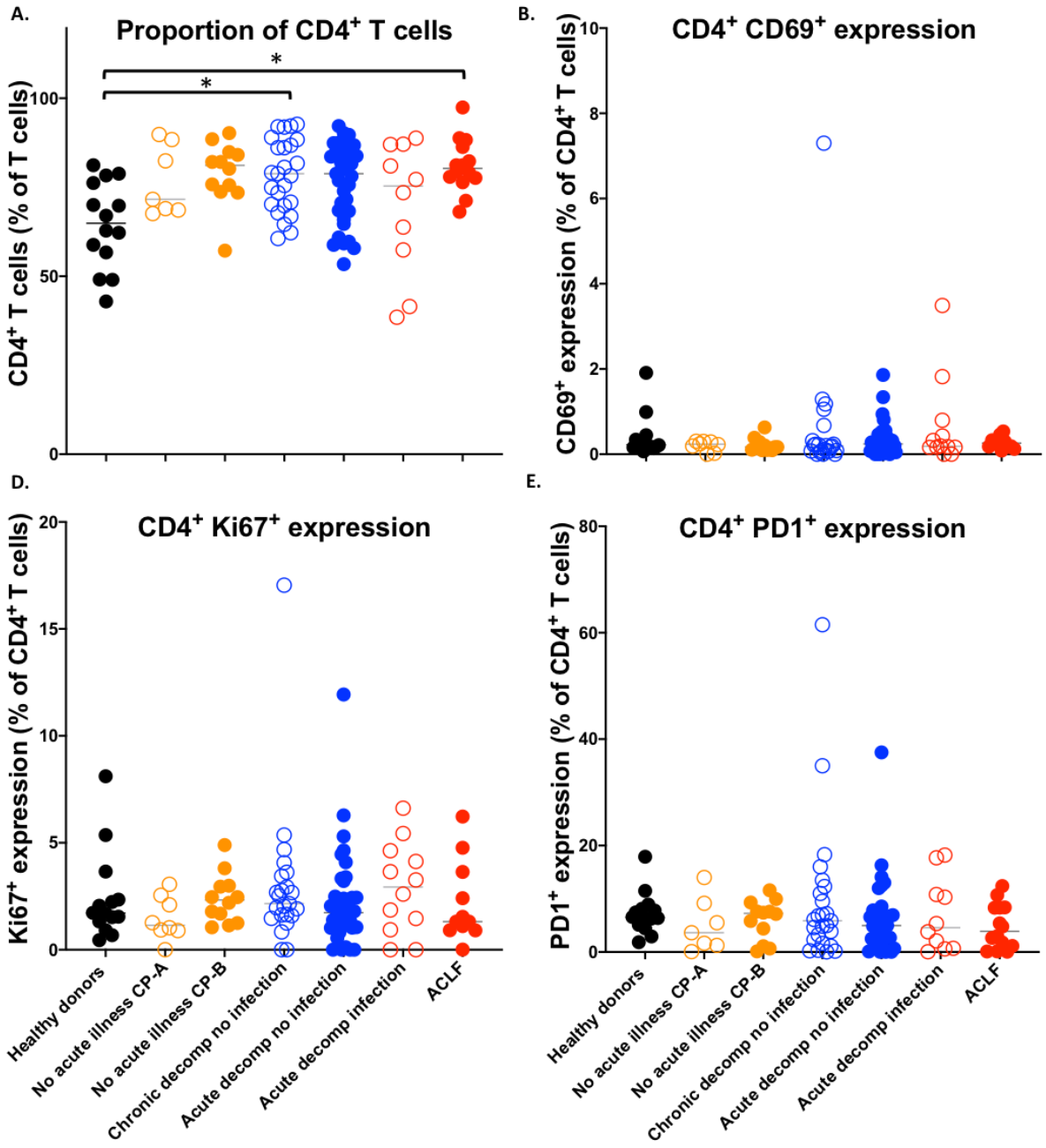

Figure 6 Conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 115 liver disease patients and 16 healthy donors

Comparisons were made against (A) the proportion of CD4+ T cells within all T cells and the expression of the activation markers (C) CD69, (D) Ki67 and (E) PD1 on CD4+ T cells across patient groups: no acute illness (CP-A), no acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Kruskall-Wallis and Dunn's multiple comparison tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and the horizontal line represents the median value.

$CD8^+$ T cells

The proportion of T cells which were $CD8^+$ and the markers $CD69^+$, $Ki67^+$ and $PD1^+$ were measured. The proportion of $CD8^+$ T cells were significantly reduced in patients with no acute illness (CP-B,) (p=0.0062), chronic decompensation (no infection, p=0.0129), acute decompensation (no infection, p=0.0409) and ACLF (p=0.0063) compared with healthy donors (Figure 7A). There was no difference in functional markers of $CD8^+$ T cells across participant groups (Figures 7B, 7C and 7D).

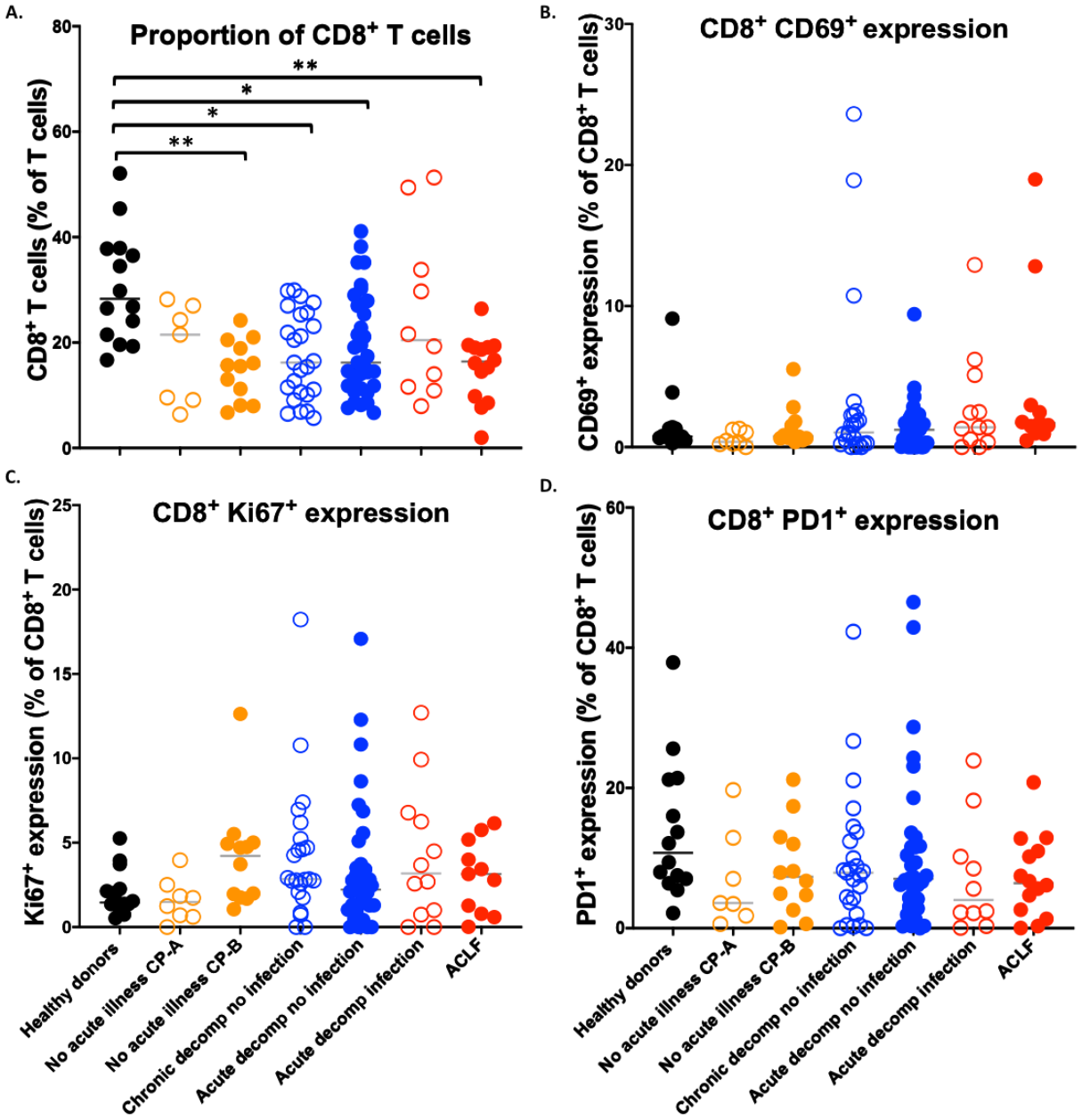

Figure 7 Conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 115 liver disease patients and 16 healthy donors

Comparisons were made against (A) the proportion of $CD8^+$ T cells within all T cells and the expression of the activation markers (C) CD69, (D) Ki67 and (E) PD1 on $CD8^+$ T cells across patient groups: no acute illness (CP-A), no acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Kruskall-Wallis and Dunn's multiple comparison tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and the horizontal line represents the median value.

Non-conventional T cells

The proportion of NKT cells ($CD56^+$ T cells), γδ T cells and MAIT cells ($V\alpha7.2^+$ $CD161^+$ T cells) were measured (Figure 8). Figure A shows acute decompensation (no infection) patients had significantly higher proportions of NKT cells in comparison with healthy donors (p=00378). No differences were observed in the proportion of γδ T cells across participant groups (Figure B). The proportion of MAIT cells was significantly reduced in patients with no acute illness (CP-B), acute decompensation (no infection) and ACLF compared to healthy donors (p=0.0031, <0.0001 and 0.0065, respectively; Figure 8C). The proportion of MAIT cells was also significantly lower in acute decompensation (no infection) patients compared to patients with chronic decompensation (no infection) and ACLF (p=0.0158 and 0.0404, respectively).

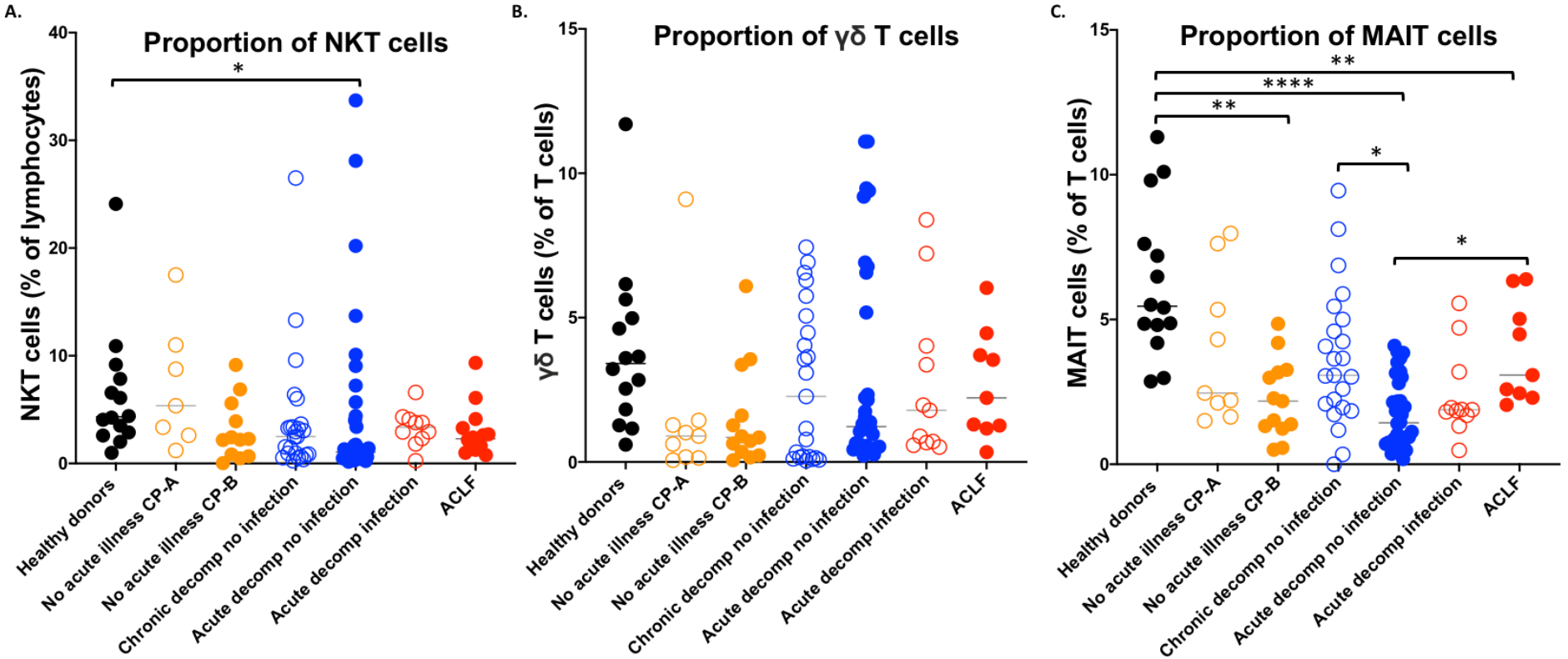

Figure 8 Non-conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 115 liver disease patients and 16 healthy donors

Comparisons were made against (A) the proportion of NKT T cells within all live lymphocytes, (B) the proportion of γδ within all T cells and (C) the proportion of MAIT cells within all T cells across patient groups: no acute illness (CP-A), no acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Kruskall-Wallis and Dunn's multiple comparison tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and the horizontal line represents the median value.

Despite no differences in total lymphocyte count, ACLF and chronic decompensated patients were associated with increased proportions of $CD4^+$ T cells. No acute illness (CP-B), chronic decompensation (no infection), acute decompensation (no infection) and ACLF were also characterised by a reduction in $CD8^+$ T cells. This reiterates the importance of analysing individual lymphocyte subsets across patient groups. No acute illness (CP-B), acute decompensation (no infection) and ACLF were all characterised by a reduction in MAIT cells, signifying the role of MAIT cells in immune dysregulation. In contrast, NK cells appear to have little importance in sterile inflammation of liver disease.

4.4 Paired peripheral blood and ascites samples

A common secondary manifestation of cirrhosis is ascites, which as previously stated is associated with poor 5-year relative survival rates. Furthermore, cirrhosis is associated with increased susceptibility to infection. Patients with ascites are at a high risk to developing SBP, which without intervention can result in renal failure. Therefore, it is pivotal to gain understanding of the immune profile of ascitic fluid and differentiate between compartments, particularly in patients with detected SBP. Peripheral blood and ascites were taken from patients attending elective paracentesis or acute admission (Table 6).

Table 6 Patients with Paired Peripheral Blood and Ascites

		All patients	Chronic decomp (no infection)	Acute decomp (no infection)	Acute decomp (infection)	ACLF
Sex	Male	**22** (75.86%)	**10** (76.92%)	**5** (71.43%)	**4** (100%)	**3** (60%)
	Female	**7** (24.14%)	**3** (23.08%)	**2** (28.57%)	---	**2** (40%)
Age	20-29	---	---	---	---	---
	30-39	**1** (3.45%)	---	---	---	**1** (20%)
	40-49	**1** (3.45%)	---	---	---	**1** (20%)
	50-59	**9** (31.03%)	**6** (46.15%)	**1** (14.29%)	**1** (25%)	**1** (20%)
	60-69	**9** (31.03%)	**2** (15.38%)	**3** (42.86%)	**2** (50%)	**2** (40%)
	70-79	**9** (31.03%)	**5** (38.46%)	**3** (42.86%)	**1** (25%)	---
	80+	---	---	---	---	
Aetiology	ALD	**24** (82.76%)	**11** (84.62%)	**6** (85.71%)	**2** (50%)	**5** (100%)
	NASH	**3** (10.34%)	**2** (15.38%)	---	**1** (25%)	---
	ALD/ HCV	**1** (3.45%)	---	**1** (14.29%)	---	---
	Cryptogenic	**1** (3.45%)	---	---	**1** (25%)	---
	AIH	---	---	---	---	---
	Cardiac	---	---	---	---	---
Cause of admission	Ascites	**8** (27.56%)	**4** (30.77%)	**4** (57.14%)	---	---
	Variceal bleeding	**1** (3.45%)	---	**1** (14.29%)	---	---
	Non-SBP infection	**5** (17.24%)	---	---	**2** (50%)	**3** (60%)
	Ascites EP	**9** (31.03%)	**9** (69.23%)	---	---	---
	No acute illness CP-B	---	---	---	---	---
	Alcoholic hepatitis	**2** (6.90%)	---	**1** (14.29%)	---	**1** (20%)
	No acute illness CP-A	---	---	---	---	---
	HE	---	---	---	---	---
	Fall/fit	---	---	---	---	---
	Non variceal UGI bleeding	**1** (3.45%)	---	**1** (14.29%)	---	---
	SBP	**3** (10.34%)	---	---	**2** (50%)	**1** (20%)
	Lymphoma	---	---	---	---	---
	Jaundice/ synthetic failure	---	---	---	---	---
Child-Pugh Score	A	---	---	---	---	---
	B	**9** (31.03%)	**7** (53.85%)	**1** (14.29%)	**1** (25%)	---
	C	**20** (68.97%)	**6** (46.15%)	**6** (85.71%)	**3** (75%)	**5** (100%)
Total		**29**	**13**	**7**	**4**	**5**

Of the 115 patients, peripheral blood and ascitic fluid were obtained from 29 patients. This cohort predominantly consisted of males (75.9%) with a median age of 63, which was similar to the total population recruited. 82.8% of these patients had ALD, followed by NASH (10.3%), ALD with concurrent HCV infection (35%) and cryptogenic liver disease (3.5%). The majority of patients recruited had attended University Hospital of Wales for elective paracentesis (31%) or were admitted with ascites (27.6%). Other causes of admission were due to infection, 17.2% suffered from non-SBP infections and 10.3% had SBP, 6.9% of patients were admitted with alcoholic hepatitis and 3.5% of patients had variceal and non-variceal UGI bleeding as the primary cause of admission. This population predominantly had CP-C (69%) with the remaining population having CP-B. Patients were categorized into chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and ACLF as described above.

Chronic decompensation (no infection)

Paired peripheral blood and ascites were obtained from 13 patients with chronic decompensation (no infection). 76.9% were male with ages ranging from 50-76 years, and a median age of 64. A large proportion of patients had ALD (84.2%) with the remaining patients having NASH. Nine patients were recruited whilst attending for elective paracentesis, whilst 4 patients were recruited following admission due to ascites. 53.9% of patients had CP-B and 46.2% of patients had CP-C.

Acute decompensation (no infection)

5 males and 2 females, with a median age of 67 years (ages ranged from 56-76) were recruited with acute decompensation (no infection). 85.7% of patients had ALD and one patient had ALD with concurrent HCV infection (14.3%). Patients recruited with admitted with ascites (57.1%), alcoholic hepatitis (14.3%), variceal bleeding (14.3%) and non-variceal UGI bleeding (14.3%). 85.7% of patients had CP-C, and one patient had CP-B.

Acute decompensation (infection)

Peripheral blood and ascites were obtained from 4 males with ages ranging from 54-72 years (median age of 64) with acute decompensation (infection). The aetiologies of this cohort were ALD (50%), NASH (25%) and cryptogenic liver disease (25%). Patients were admitted having either SBP (50%) or non-SBP infections (50%). Three patients had CP-C, whilst 1 patient had CP-B.

ACLF

Three males and 2 females, with ALD and CP-C, were recruited with ACLF. Age of patients ranged from 33-63, with patients having a markedly lower median age compared to other groups (55 years). 60% of patients were admitted presenting with non-SBP infections, 20% had SBP and 20% had alcoholic hepatitis.

SBP

There were only 3 individuals with active SBP infection. These were grouped in the above categories and highlighted in red in the figures to ensure that each group had at least 5 paired samples for statistical analysis. When analysed as a separate group there was not a distinct profile that separated SBP from other individuals.

Comparison of cellular phenotype and functional marker profiles of peripheral blood and ascites

To compare immune cell phenotype and functional marker profiles of peripheral blood and ascites of cirrhotic patients, conventional and non-conventional lymphocytes were isolated and analysed by multiparameter flow cytometry. Patients were stratified into chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and ACLF.

NK cells

In concordance with previous analysis, the proportion of NK cells, the proportion of CD56 bright NK cells and the expression of the activation markers CD69, Ki67 and PD1 expression on NK cells was measured (Figure 9). The proportion of NK cells was increased in ascites compared to peripheral blood in chronic decompensated (no infection) and ACLF patients (p=0.0017 and

0.0625, respectively; Figure 9A). No significant differences were observed in the proportion of CD56 bright NK cells with the NK population across patient groups (Figure 9B). Ascites NK $CD69^+$ expression was elevated in ACLF patients (p=0.0625) compared to peripheral blood, as shown in Figure 9C. Conversely, ascites NK $Ki67^+$ expression was significantly reduced in chronic decompensated (no infection) patients (p=0.0012) and ACLF (p=0.0625) compared to peripheral blood (Figure 9D). The expression of NK $PD1^+$ was significantly elevated in the ascites of acute decompensation (no infection) patients in comparison with peripheral blood (p=0.046), as demonstrated in Figure 9E.

Patients with SBP (highlighted in red) were associated with increased proportions of NK cells which had elevated expression of CD69 and reduced Ki67 expression in the ascites compared to peripheral blood.

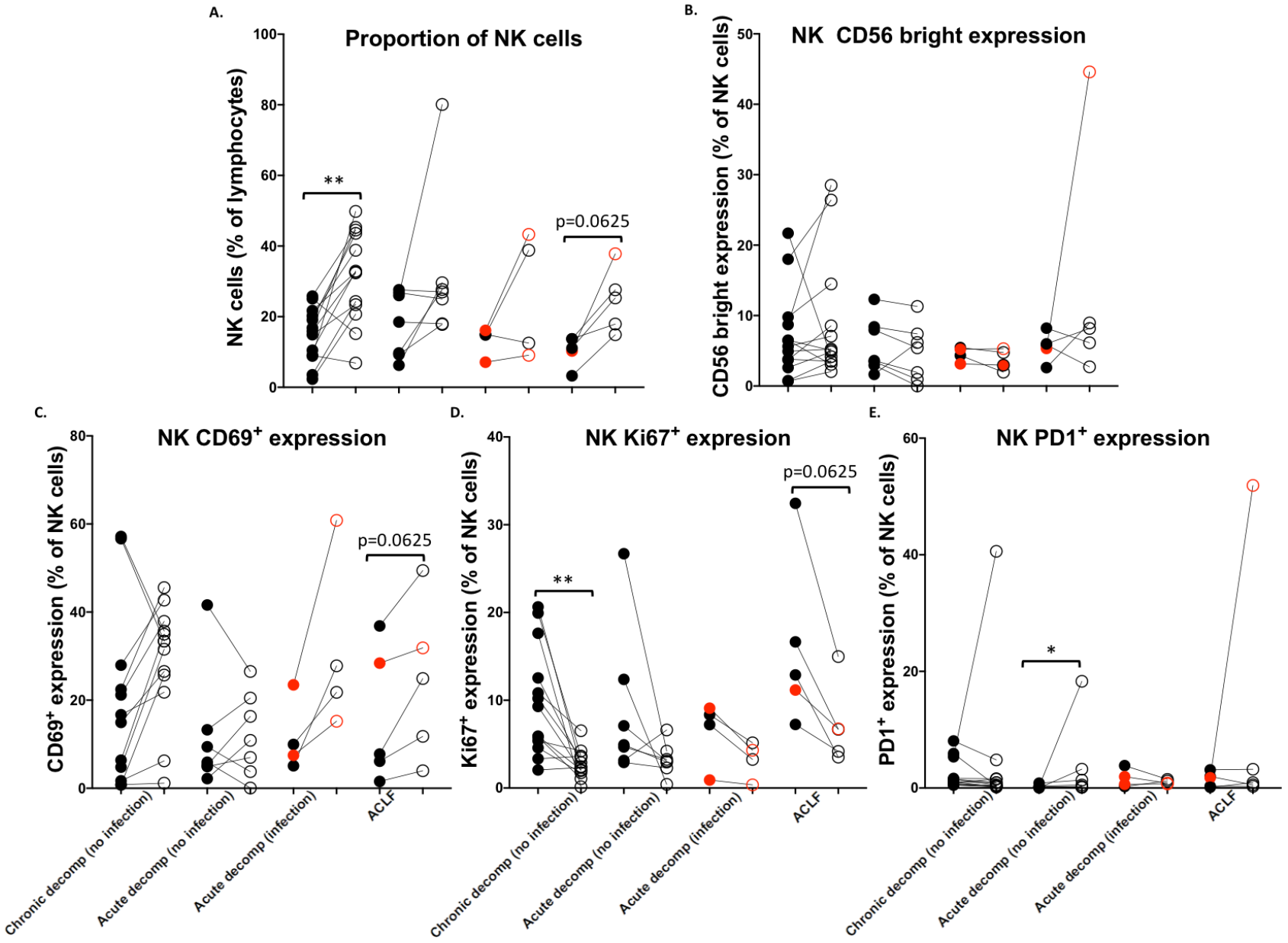
A.
Proportion of NK cells
NK cells (% of lymphocytes)
**
p=0.0625
B.
NK CD56 bright expression
CD56 bright expression (% of NK cells)
C.
NK CD69+ expression
CD69+ expression (% of NK cells)
p=0.0625
D.
NK Ki67+ expresion
Ki67+ expression (% of NK cells)
**
p=0.0625
E.
NK PD1+ expression
PD1+ expression (% of NK cells)
*
Chronic decomp (no infection)
Acute decomp (no infection)
Acute decomp (infection)
ACLF

Figure 9 Conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 29 patients with paired peripheral blood and ascites

Comparisons were made against (A) the proportion of NK cells within all live lymphocytes, (B) the proportion CD56 bright NK cells within the NK cell population and the expression of the activation markers (C) CD69, (D) Ki67 and (E) PD1 on NK cells across patient groups: chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Wilcoxon matched pairs signed rank tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and red represents patients with SBP

$CD4^+$ T cells

The proportion $CD4^+$ T cells within the overall T cell population and markers including CD69, Ki67 and PD1 were compared between peripheral blood and ascites (Figure 10). The proportion of $CD4^+$ T cells were reduced in the ascites of chronic decompensation (no infection), acute decompensation (no infection) and ACLF patients (p=0.0002, 0.0156 and 0.0625, respectively) compared to peripheral blood (Figure 10A). Ascites $CD4^+$ $CD69^+$ expression was significantly elevated in chronic decompensated patients (p=0.0002, Figure 10B). No significant differences were observed between peripheral blood and ascites $CD4^+$ $Ki67^+$ expression across patient groups (Figure 10C). $CD4^+$ $PD1^+$ expression was increased in the ascites of chronic decompensation (no infection) (p=0.0002) and ACLF patients (p=0.0625) compared to peripheral blood, as shown in Figure 10D.

Participants with SBP (in red) were characterised by a reduced proportion of $CD4^+$ T cells in the ascites compared to peripheral blood (Figure 10A). CD69 expression was increased in acute decompensated (infection) but reduced in ACLF patients. This may be a reflection of the immune dysfunction associated with ACLF. PD1 expression of $CD4^+$ T cells was also increased in ascites with SBP compared to peripheral blood (Figure 10D).

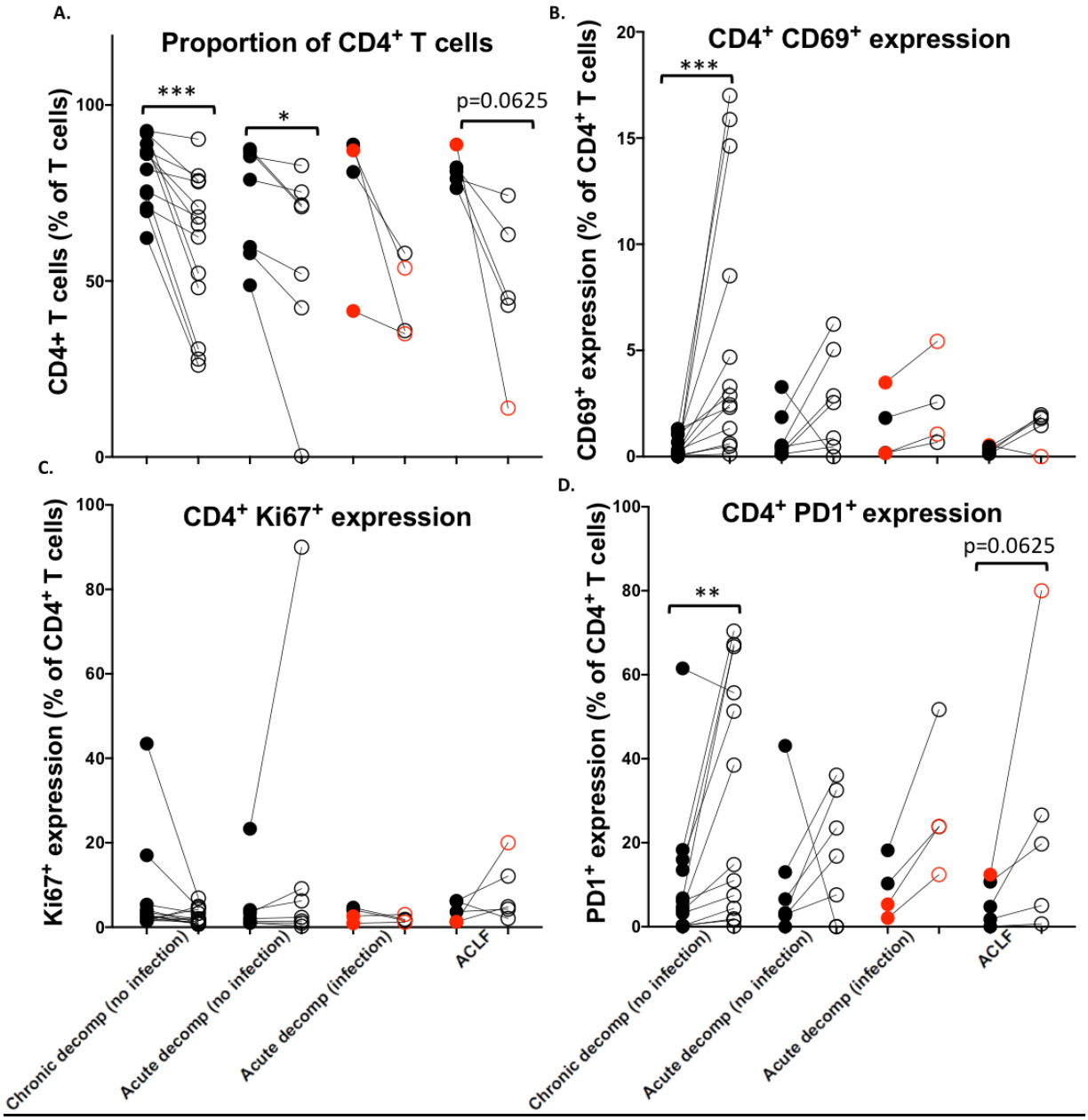

Figure 10 Conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 29 patients with paired peripheral blood and ascites

Comparisons were made against (A) the proportion of $CD4^+$ T cells within all T cells and the expression of the activation markers (C) CD69, (D) Ki67 and (E) PD1 on $CD4^+$ T cells across patient groups: chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Wilcoxon matched pairs signed rank tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and red represents patients with SBP.

$CD8^+$ T cells

The proportion of T cells which were $CD8^+$ and markers including CD69, Ki67 and PD1 were measured (Figure 11). Chronic decompensation (no infection) and ACLF patients had a greater proportion of $CD8^+$ T cells observed in ascites compared to peripheral blood (p=0.0002 and 0.0625, respectively, Figure 11A). Ascites $CD8^+$ $CD69^+$ expression was significantly elevated in chronic decompensation (no infection) and acute decompensation (no infection) patients compared to peripheral blood (p=0.0002 and 0.0156, respectively), as shown in Figure 11B. Chronic decompensated patients were also characterised by reduced $CD8^+$ $Ki67^+$ expression in ascites when compared with peripheral blood (p=0.0002, Figure 11C). Expression of $CD8^+$ $PD1^+$ was increased in the ascites of chronic decompensation (no infection) patients (p=0.0002) and ACLF patients (p=0.0625) compared to paired peripheral blood (Figure 11D).

The 3 participants with SBP infections had increased proportions of $CD8^+$ T cells with elevated expression of PD1 in the ascites compared to peripheral blood. (Figures 11A and 11D). Similar to $CD4^+$ subset, CD69 expression was increased in ascites compared to peripheral blood in acute decompensation (infection) patients with SBP, but not ACLF

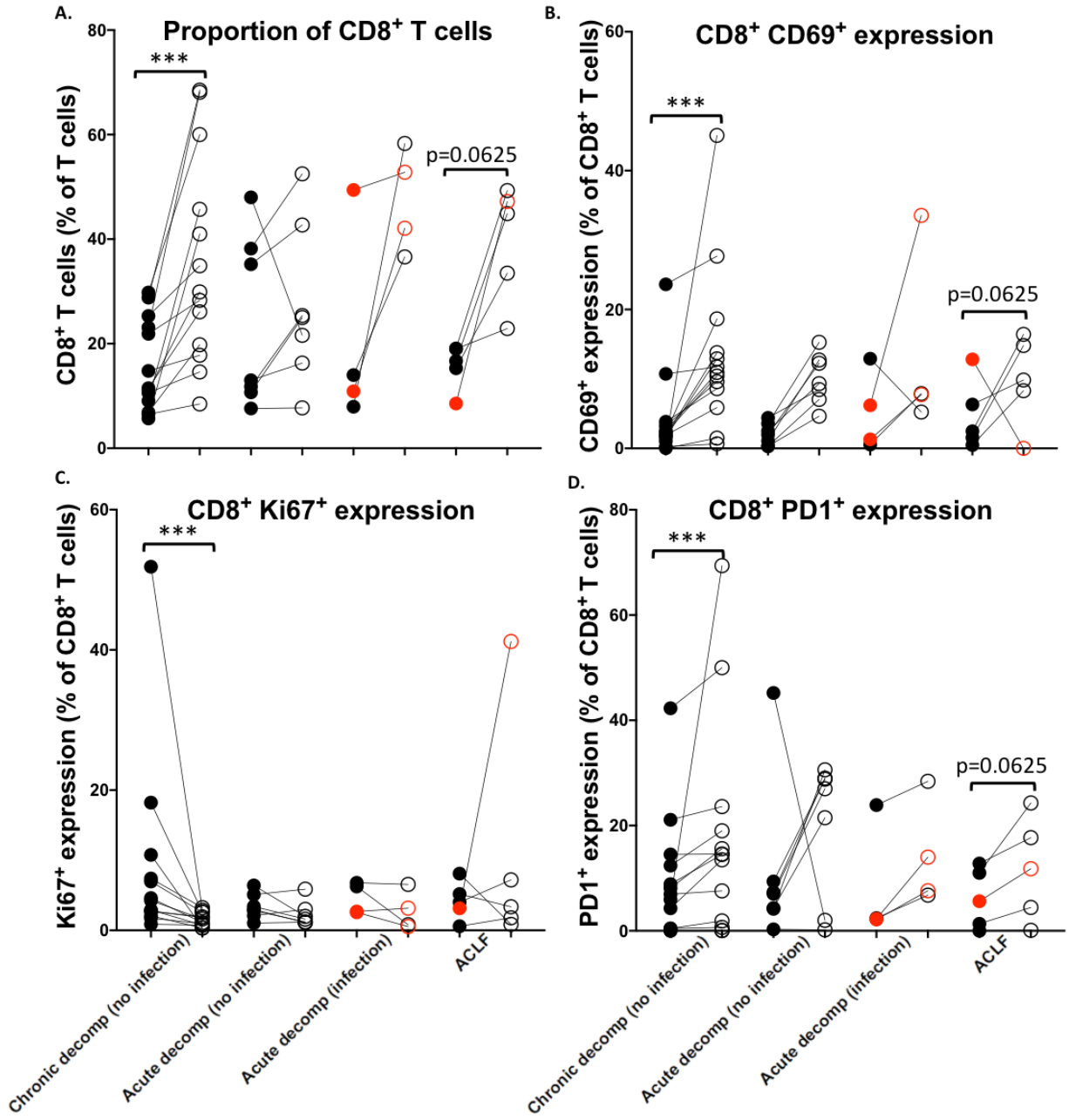

Figure 11 Conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 29 patients with paired peripheral blood and ascites

Comparisons were made against (A) the proportion of $CD8^+$ T cells within all T cells and the expression of the activation markers (C) CD69, (D) Ki67 and (E) PD1 on $CD8^+$ T cells across patient groups: chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Wilcoxon matched pairs signed rank tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and red represents patients with SBP.

Non-conventional T cells

The proportion of NKT cells ($CD56^+$ T cells), γδ T cells and MAIT cells ($V\alpha7.2^+$ $CD161^+$ T cells) were measured in paired samples (Figure 12). The proportion of NKT cells was elevated in ascites of chronic decompensation (no infection) and ACLF patients (p=0.0061 and 0.0625, respectively, Figure 12A). No differences were observed in the proportions of γδ T cells and MAIT cells between compartments (Figures 12B and 12C).

No obvious trends were observed in the proportion of non-conventional T cells between compartments of individuals with SBP infection.

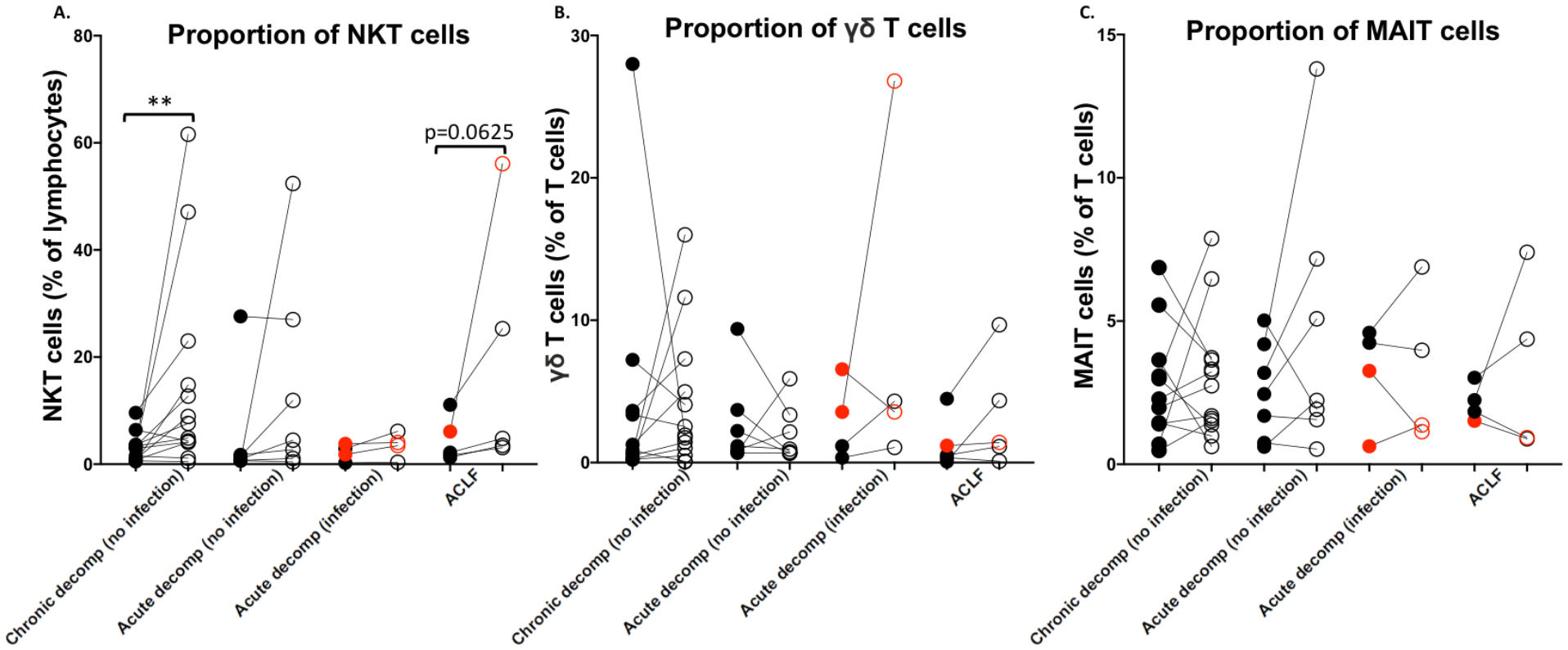

Figure 12 Non-conventional lymphocytes were isolated and analysed by multiparameter flow cytometry for 29 patients with paired peripheral blood and ascites

Comparisons were made against (A) the proportion of NKT T cells within all live lymphocytes, (B) the proportion of γδ T cells within all T cells and (C) the proportion of MAIT cells within all T cells across patient groups: chronic decompensation (no infection), acute decompensation (no infection), acute decompensation (infection) and acute-on-chronic liver failure (ACLF). Statistical comparisons were made using Wilcoxon matched pairs signed rank tests. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and red represents patients with SBP.

Ascites had a distinctly different immune profile in comparison with peripheral blood. Ascites lymphocyte profile was characterised by increased NK, $CD8^+$ T cell and NKT populations, and a decreased proportion of $CD4^+$ T cells. Ascites PD1 expression was elevated in CD4+ and CD8+ T cell subsets. This may imply T cells in the ascites are exhausted, contributing to immune dysregulation and increased susceptibility of SBP. Patients with detected SBP infection were also associated with increased PD1 expression in both $CD4^+$ and $CD8^+$ T cell subsets.

In summary, my findings confirm that ACLF has a defined clinical and cellular immune profile. Despite no differences observed in the total lymphocyte count, CD4+ and CD8+ subsets and expression of activation markers were different across patient groups. The proportions of MAIT cells were markedly different between patient groups, suggesting their contribution to immune dysregulation in cirrhosis. Ascites demonstrated a unique immune profile in comparison to peripheral blood. In particular PD1 expression was significantly increased. This accompanied by increased CD69 expression suggests T cell activation. Overexpression of PD1 is a definitive characteristic of T cell exhaustion (Jubel et al., 2020). Elevated PD1 may therefore contribute to immune dysregulation and increased susceptibility to SBP infections. Function T cell assays will be required to determine the status of T cells in ascites. Only 3 patients recruited had detected SBP infection, therefore a statistically accurate interpretation of immune profiles associated with SBP is not possible. There was not a distinct profile of the SBP samples however, they frequently included lymphocyte phenotypes which were more marked reflecting the acute inflammatory response.

5. Luminex Multiplex Analysis

Luminex multiplex assays were conducted in order to gain insight into the humoral response in decompensated cirrhosis. Soluble mediators are produced by local tissues and activated immune cells to orchestrate an inflammatory response. Luminex multiplex assays allow for simultaneous detection of a broad range of analytes for a given sample. An unbiased range of markers was selected in order to reduce prejudice of which analytes may be important and which not in liver disease.

Luminex assays were performed on two separate occasions. The initial Luminex assay was performed on 20 samples in order to test different dilutions and determine appropriate experimental conditions for the remaining samples. There was not published literature experience on the use of Luminex to assess ascitic chemokines and cytokines at the time of this experiment. Samples were tested at 1:2 and 1:10 dilutions. Samples with 1:2 dilutions performed best as a greater percentage of analyte concentrations fell within the detection range and therefore were carried through for the remainder of the project. In the second instance, a further 19 patients and 4 healthy donors were analysed making a total of 39 patient samples and 4 healthy donors, across the two experiments.

5.1 Patient Demographics

Healthy donors

Four healthy donors recruited through the 'Coordinating COVID-19 asymptomatic testing programmes in university settings: providing insight on acquired immunity across the student population' study, were analysed by Luminex. Blood was taken from 2 males and 2 females with ages ranging from 25-50 years.

No acute illness (CP-A)

One male, aged 65 with ALD, was included in the preliminary Luminex experiments.

No acute illness (CP-B)

Three males, with CP-B aged between 56-78 (median age: 58), were recruited with no acute illness (CP-B). One patient had ALD, and the remaining 2 patients had NASH.

Chronic decompensation (no infection)

This subgroup consisted of 3 females and 7 males aged between 44-75 (median age of 56) with chronic decompensation (no infection). The majority of patients had ALD (80%), whilst 20% had NASH. 80% of patients were recruited following elective paracentesis, 10% were admitted with ascites and 10% were categorised under fall/fit. Over half of the patients had CP-C (60%), and 40% were CP-B.

Acute decompensation (no infection)

Fifteen patients with acute decompensation (no infection) were included in the Luminex analysis. Nine of which were male and 5 females with a median age of 62 (age ranged from 45-76). 73.3% of patients had ALD, 20% had NASH and 1 patient had ALD with concurrent HCV infection (6.7%). Patients were admitted due to ascites (33.3%), non-variceal UGI bleeding (13,3%), HE (20%), alcoholic hepatitis (13.3%), variceal bleeding (13.3%) and fall/fit (6,7%). Patients were predominately CP-C (80%), and 20% were CP-B.

Acute decompensation (infection)

One patient with SBP and 2 patients with non-SBP infections were used for Luminex analysis. This group consisted of 3 males aged between 48-63 (median 54) with ALD (67%) or Cryptogenic (33%). All patients had CP-C.

ACLF

Seven patients with ACLF were involved in initial Luminex assays. These included 2 females and 5 males with a median aged of 61 (ranging from 45-64). All patients had ALD, with one patient having concurrent HCV infection. Patients were admitted, and subsequently recruited, due to variceal bleeding (14.3%), alcoholic hepatitis (28.6%), SBP infection (28.6%), non-SBP infection and jaundice (14.3%). One patient was CP-B, with the remaining patients having CP-C.

To compare humoral profiles of plasma and ascites of cirrhotic patients, the concentration of 31 analytes was analysed in both compartments by Luminex multiplex analysis (Table 7).

Table 7 Patient Demographics for Luminex Analysis

		All patients	Healthy Donors	No acute illness (CP-A)	No acute illness (CP-B)	Chronic decomp (no infection	Acute decomp (no infection	Acute decomp (infection)	ACLF
Sex	Male	**28** (71.8%)	**2** (50%)	**1** (100%)	**3** (100%)	**7** (70%)	**9** (60%)	**3** (100%)	**5** (71.4%)
	Female	**11** (28.2%)	**2** (50%)	---	---	**3** (30%)	**6** (40%)	---	**2** (28.6%)
Age	20-29	---		---	---	---	---	---	---
	30-39	---		---	---	---	---	---	---
	40-49	**7** (17.9%)		---	---	**3** (30%)	**2** (13.3%)	**1** (33.3%)	**1** (14.3%)
	50-59	**11** (28.2%)		---	**2** (66.7%)	**3** (30%)	**4** (26.7%)	**1** (33.3%)	**1** (14.3%)
	60-69	**15** (38.5%)		**1** (100%)	---	**1** (10%)	**7** (46.7%)	**1** (33.3%)	**5** (71.4%)
	70-79	**6** (15.4%)		---	**1** (33.3%)	**3** (30%)	**2** (13.3%)	---	---
	80+	---		---	---	---	---	---	---
Aetiology	ALD	**29** (74.4%)		**1** (100%)	**1** (33.3%)	**8** (80%)	**11** (73.3%)	**2** (66.7%)	**6** (85.7%)
	NASH	**7** (17.9%)		---	**2** (66.7%)	**2** (20%)	**3** (20%)	---	---
	ALD/HCV	**2** (5.1%)		---	---	---	**1** (6.7%)	---	**1** (14.3%)
	Cryptogenic	**1** (2.6%)		---	---	---	---	**1** (33.3%)	---
	AIH	---		---	---	---	---	---	---
	Cardiac	---		---	---	---	---	---	---
Cause of admission	Ascites	**6** (15.4%)		---	---	**1** (10%)	**5** (33.3%)	---	---
	Variceal bleeding	**3** (7.7%)		---	---	---	**2** (13.3%)	---	**1** (14.3%)
	Non-SBP infection	**3** (7.7%)		---	---	---	---	**2** (66.7%)	**1** (14.3%)
	Ascites EP	**8** (20.5%)		---	---	**8** (80%)	---	---	---
	No acute illness CP-B	**3** (7.7%)		---	**3** (100%)	---	---	---	---
	Alcoholic hepatitis	**4** (10.3%)		---	---	---	**2** (13.3%)	---	**2** (28.6%)
	No acute illness CP-A	**1** (2.6%)		**1** (100%)	---	---	---	---	---
	HE	**3** (7.7%)		---	---	---	**3** (20%)	---	---
	Fall/fit	**2** (5.1%)		---	---	**1** (10%)	**1** (6.7%)	---	---
	Non variceal UGI	**2** (5.1%)		---	---	---	**2** (13.3%)	---	---
	bleeding	**3** (7.7%)		---	---	---	---	**1** (33.3%)	**2** (28.6%)
	SBP	---		---	---	---	---	---	---
	Lymphoma	**1** (2.6%)		---	---	---	---	---	**1** (14.3%)
	Jaundice/synthetic failure								
Child-Pugh Score	A	**1** (2.6%)		**1** (100%)	---	---	---	---	---
	B	**11** (28.2%)		---	**3** (100%)	**4** (40%)	**3** (20%)	---	**1** (14.3%)
	C	**27** (69.2%)		---	---	**6** (60%)	**12** (80%)	**3** (100%)	**6** (85.7%)
Total		**39**	**4**	**1**	**3**	**10**	**15**	**3**	**7**

5.2 Analytes that were preferentially enriched in Ascites Compared to Plasma

Ascites had significantly elevated levels of GM-CSF, IL-6, IL-10 and VEGF compared to plasma ($p<0.001$, 0.0008, <0.0001 and 0.00125, respectively, Figure 13). IL-6 (Figure 13B) had ascites samples which had concentrations below the lower limit of quantification (LLOQ), and plasma and ascites were below the detection limit for GM-CSF and IL-10 (Figures 13A and 13C). All concentrations were above the LLOQ for VEGF (Figure 13D). The LLOQ is derived by the concentration of the lowest kit standard. Concentrations below the standard curve cannot be extrapolated.

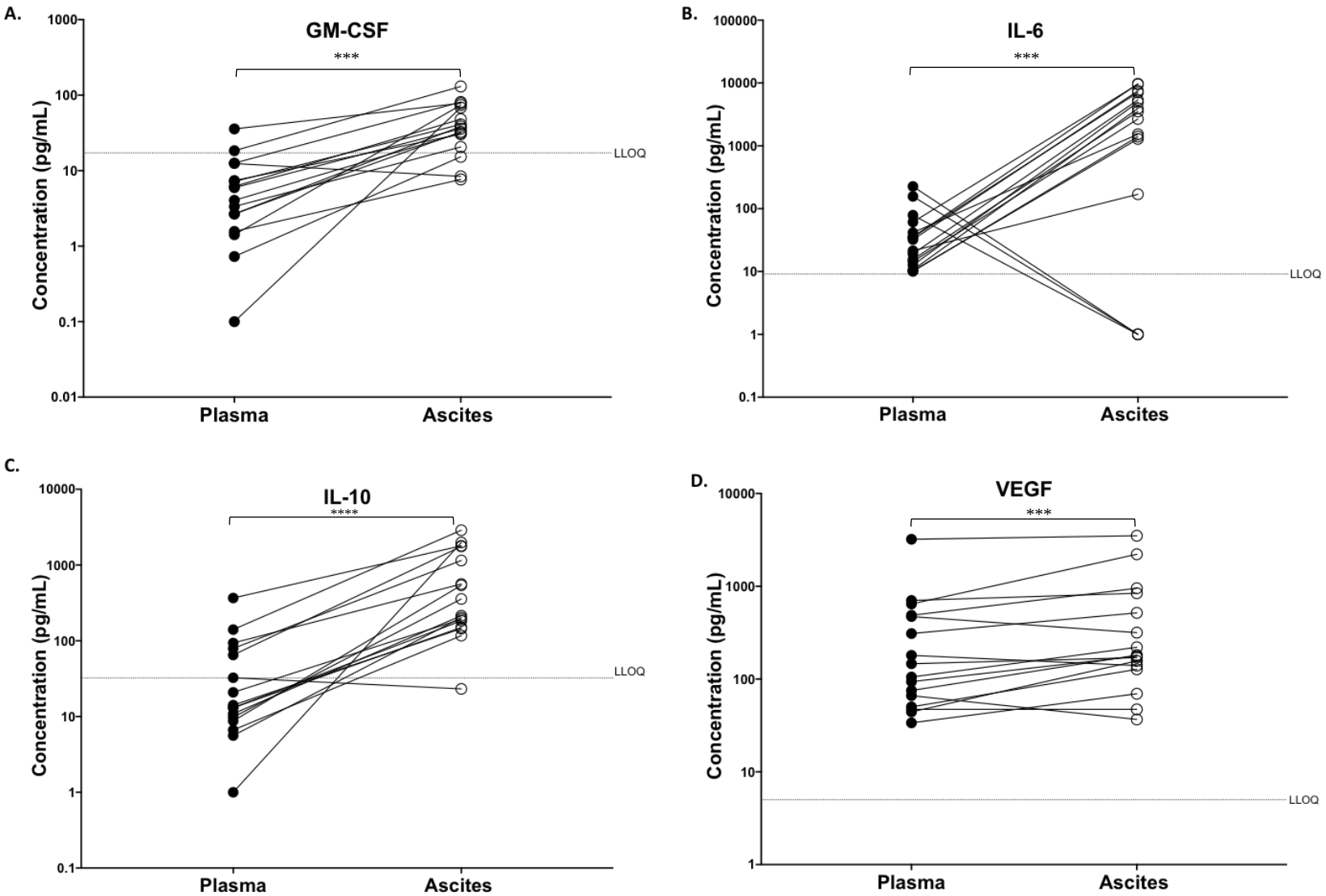

Figure 13 Paired plasma and ascites were analysed by Luminex multiplex assays for 16 patients

Comparisons were made against (A) GM-CSF, (B) IL-6 (C) IL-10 and (D) VEGF across the compartments. Statistical comparisons were made using Wilcoxon matched pairs singed rank test. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

The chemokines CCL2/MCP-1, CCL3/MIP-1α and CXCL10/IP-10 had increased concentrations in the ascites compared to plasma (p=0.0214, 0.0063 and 0.0214, respectively; Figure 14). One ascites sample was below the LLOQ for CXCL10 (Figure 14C), and concentrations of CCL3 were below the detection limit for a number of plasma and ascites samples (Figure 14B). Two distinct groups of paired samples were observed for CCL2 concentration (Figure 14A), and CXCL10 to a lesser extent (Figure 14C). This implies the two different batches (i.e. different Luminex batch and day of experiment) impacted the concentrations of analytes.

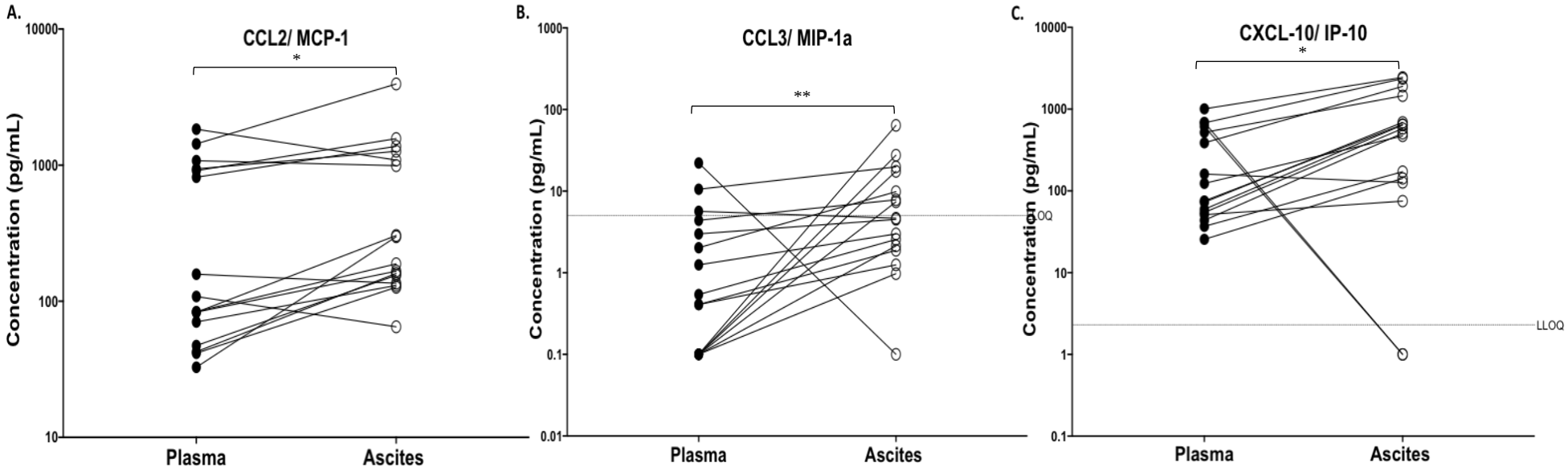

Figure 14 Paired plasma and ascites were analysed by Luminex multiplex assays for 16 patients

Comparisons were made against (A) CCL2/MCP-1, (B) CCL3/ MIP-1α and (C) CXCL10/ IP-10 across the compartments. Statistical comparisons were made using Wilcoxon matched pairs singed rank test. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.3 Analytes whose concentrations were Lower in Ascites Compared to Plasma

Concentrations of PDGF-AA, PDGF-AB/BB, PD-L1, Fas ligand, ICAM/CD54 and IL-12/IL-23p40 were significantly lower in ascites compared to paired plasma ($p=0.0386$, <0.0001, 0.0042, 0.0007, <0.0001 and 0.0003, respectively; Figure 15). Plasma samples were below the LLOQ for PDGF-AA (Figure 15A) and ascites samples were below the LLOQ for PDGF-AB/BB (Figure 15B). PD-L1, Fas Ligand and IL-12 had concentrations below the detection limit for both plasma and ascites (Figures 15C, 15D and 15F). All samples had concentrations of ICAM above the detection limit (Figure 15E).

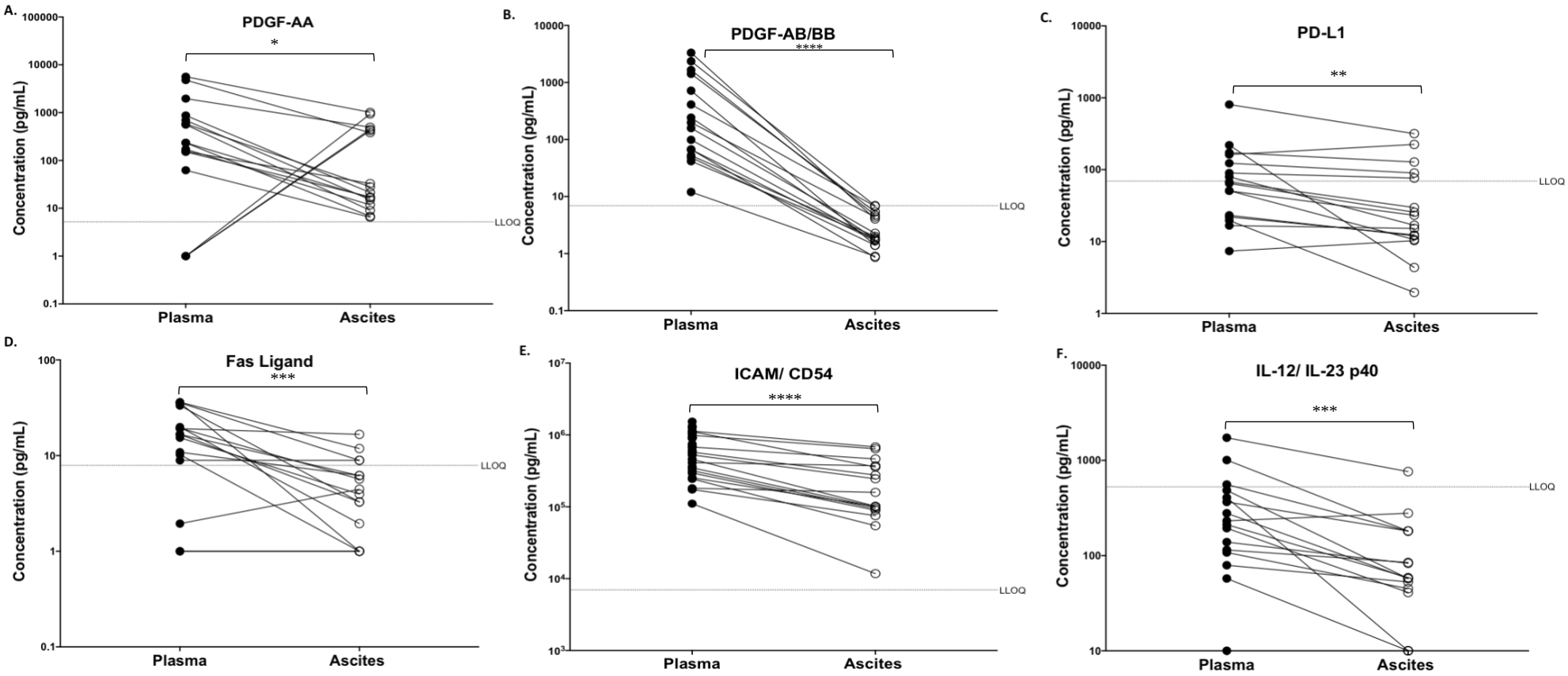

Figure 15 Paired plasma and ascites were analysed by Luminex multiplex assays for 16 patients

Comparisons were made against (A) PDGF-AA, (B) PDGF-AB/BB, (C) PD-L1, (D) Fas Ligand, (E) ICAM/CD54 and (F) IL-12/IL-23 p40 across the compartments. Statistical comparisons were made using Wilcoxon matched pairs singed rank test. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Ascites had significantly lower concentrations of the chemokines CCL5/RANTES (regulated upon activation, normal T cell expressed and secreted) and CCL4/MIP-1β compared to plasma ($p<0.0001$ and 0.0017, respectively). Ascites CCL5 levels were below the LLOQ for all samples (Figure 16A), however the majority of paired samples had concentrations below the detection limit for CCL4 (Figure 16B).

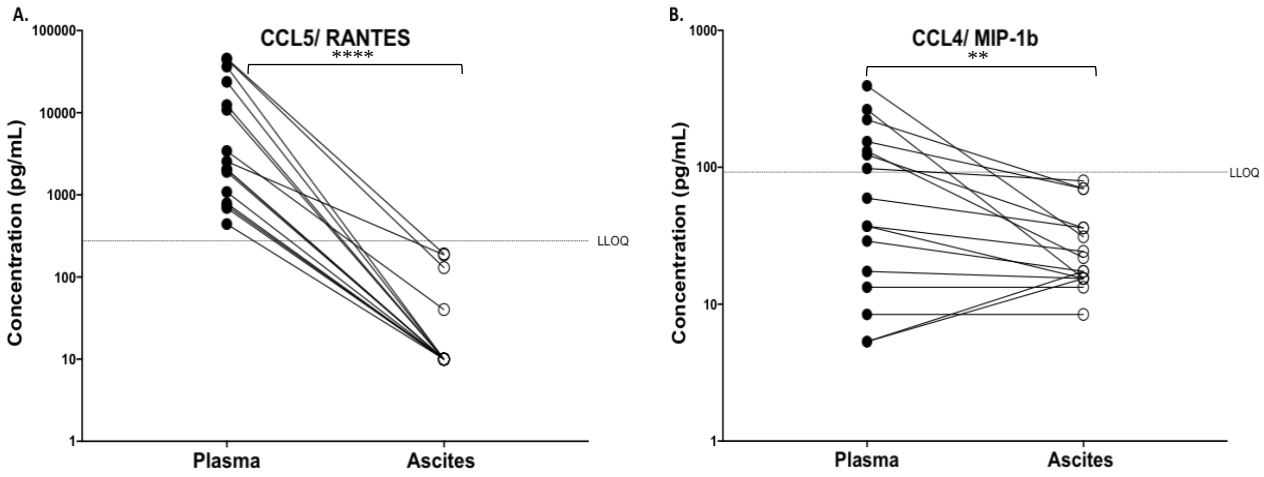

Figure 16 Paired plasma and ascites were analysed by Luminex multiplex assays for 16 patients
Comparisons were made against (A) CCL5/RANTES and (B) CCL4/MIP-1β across the compartments. Statistical comparisons were made using Wilcoxon matched pairs singed rank test. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Non-significant Analytes

No significant differences were observed in the concentration of 11 analytes between plasma and ascites, as shown in Figure. Of these analytes, batch effect presented a problem for G-CSF (Figure 17A), IL-1β (Figure 17E) and TNF-α (Figure 17G). In particular, the two batches for IL-1β demonstrated conflicting trends in terms of IL-1 β levels within the plasma and ascites.

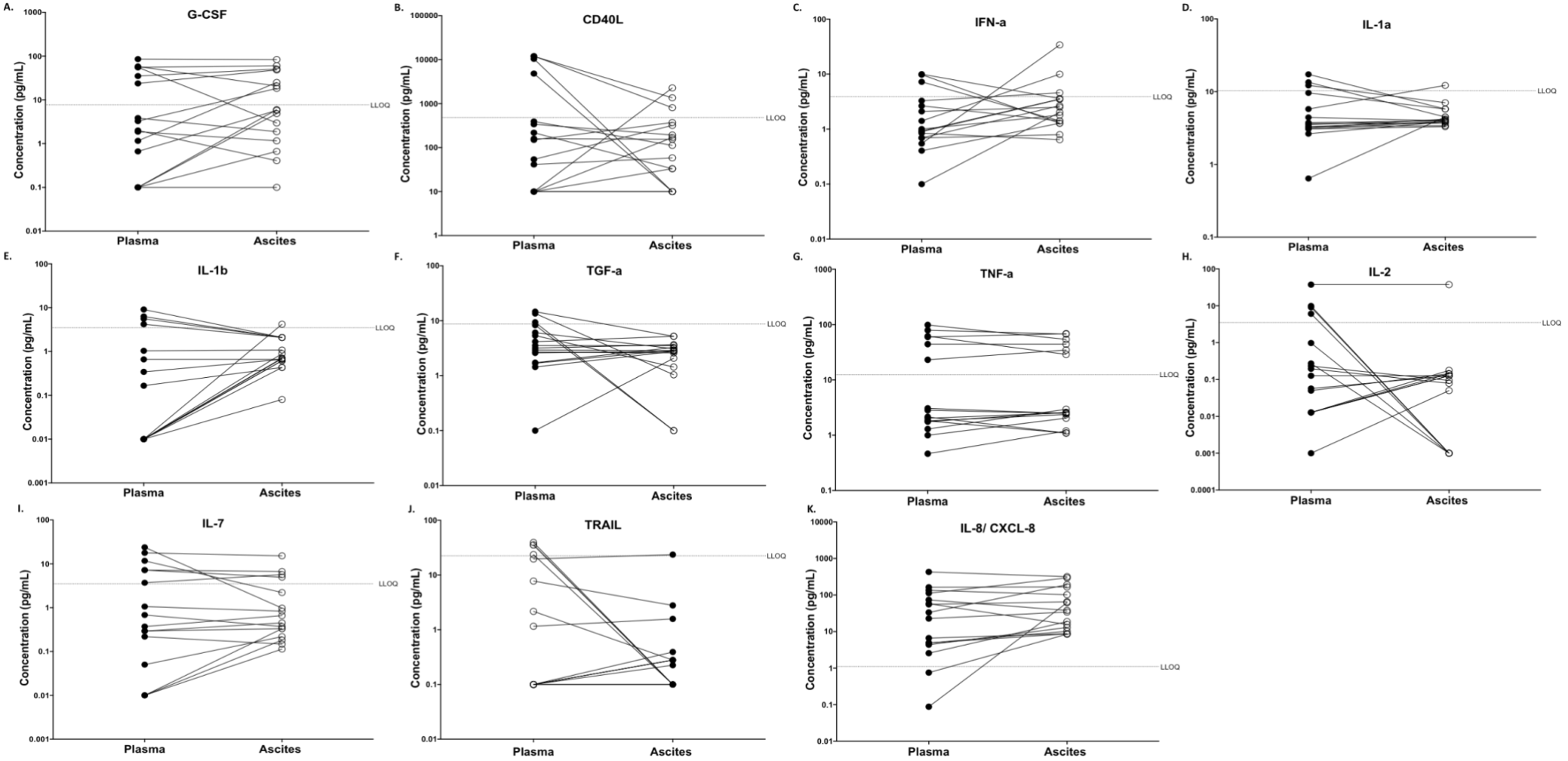

Figure 17 Paired plasma and ascites were analysed by Luminex multiplex assays for 16 patients

Comparisons were made against (A) G-CSF, (B) CD40L, (C) IFN-α, (D) IL-1α, (E) IL-1β, (F) TGF-α, (G) TNF-α, (H) IL-2, (I) IL-7, (J) TRAIL and (K) IL-8/CXCL8 across the compartments. Statistical comparisons were made using Wilcoxon matched pairs singed rank test. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Undetected Analytes

Six analytes had undetected concentrations in both plasma and ascites (Table 8). This may be due to low humoral concentrations or prove that Luminex assays are unsuitable for measuring these particular analytes.

Table 8 Undetected Analytes

Undetected Analytes
IL-17A
IFN-γ
IL-12p70
IL-4
IL-5
TNF-β

5.4 Detection Rates of Analytes

The LLOQ is defined as a measure of the lowest concentration of the analyte of interest which can be reliably quantified in a sample with a high degree of confidence. An analyte which has a 50% detection rate means that reliable concentrations were reported for 50% of patient samples. The percentage of samples which had concentrations within the detection range (as demonstrated in Figure 18A) were calculated in order to eliminate analytes where a large proportion of samples fell below the LLOQ. Analytes ranged from 1.8%-100 detected (Figure 18B). 100% of samples were within the detection range for CCL2, VEGF and ICAM (Figure 18B), this demonstrates a high degree of confidence for analyte concentrations reported. Whereas IFN-α, IFN-γ, IL-12p70, IL-4, IL-5, TNF-β and IL-12 all had detection rates below 10% (Figure 18B). One can only be confident of less than 10% of concentrations quantified for these analytes, and therefore must be eliminated from analysis. As previously stated, this may reflect the very low humoral concentrations.

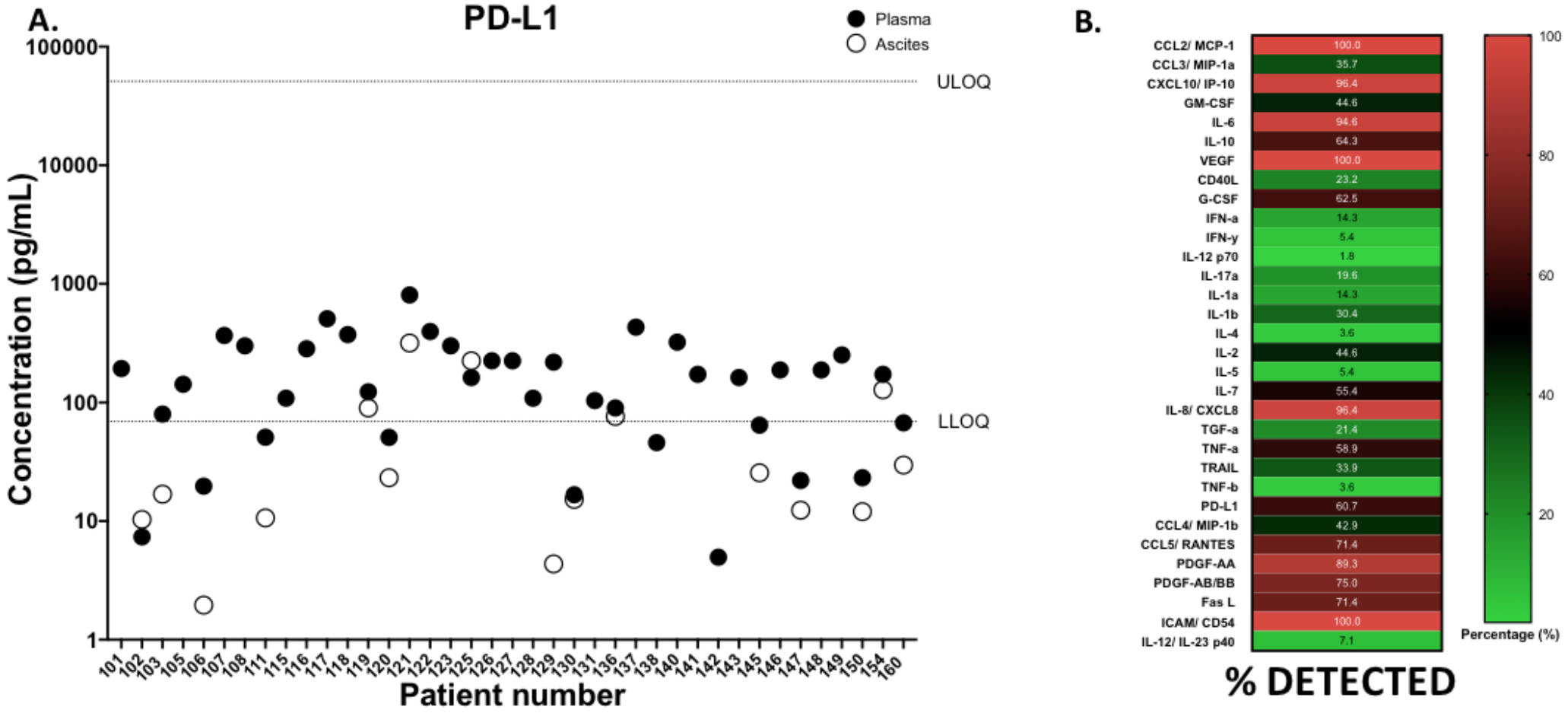

Figure 18 Plasma and ascites were analysed by Luminex multiplex assays for 39 patients and 4 healthy donors

Detected samples are those which had concentrations within the detection range i.e. between the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), shown in A. (B) shows the percentage of samples which had concentrations within the detection range for each analyte.

For accurate quantification, we identified analytes which had a detection rate greater than 50%. G-CSF, IL-7, IL-8 and TNF-α had more than 50% of samples above the LLOQ, however there was no significant difference in the analyte concentrations between plasma and ascites (Figure 19). Conversely, CCL4, CCL3, GM-CSF and IL-12 had significantly different concentrations between compartments but had less than 50% of samples within the detection range (Figure 19). The list of analytes shown in Table 9, had no significant difference in concentration between compartments and less than 50% of samples were within the detection limits. These analytes were excluded from further investigation. Ascites had significantly elevated concentrations of CXCL10, IL-10, IL-6, CCL2 and VEGF, and had more than 50% of samples above the LLOQ (Figure). Fas Ligand, PDGF-AB/BB, PDGF-AA, CCL5, PD-L1 and ICAM had significantly reduced levels in the ascites compared to plasma and had more than 50% of samples within the detection range (Figure 19). By process of elimination, these analytes should be investigated further for their importance in the pathophysiology and severity of liver disease to determine their potential as a biomarker.

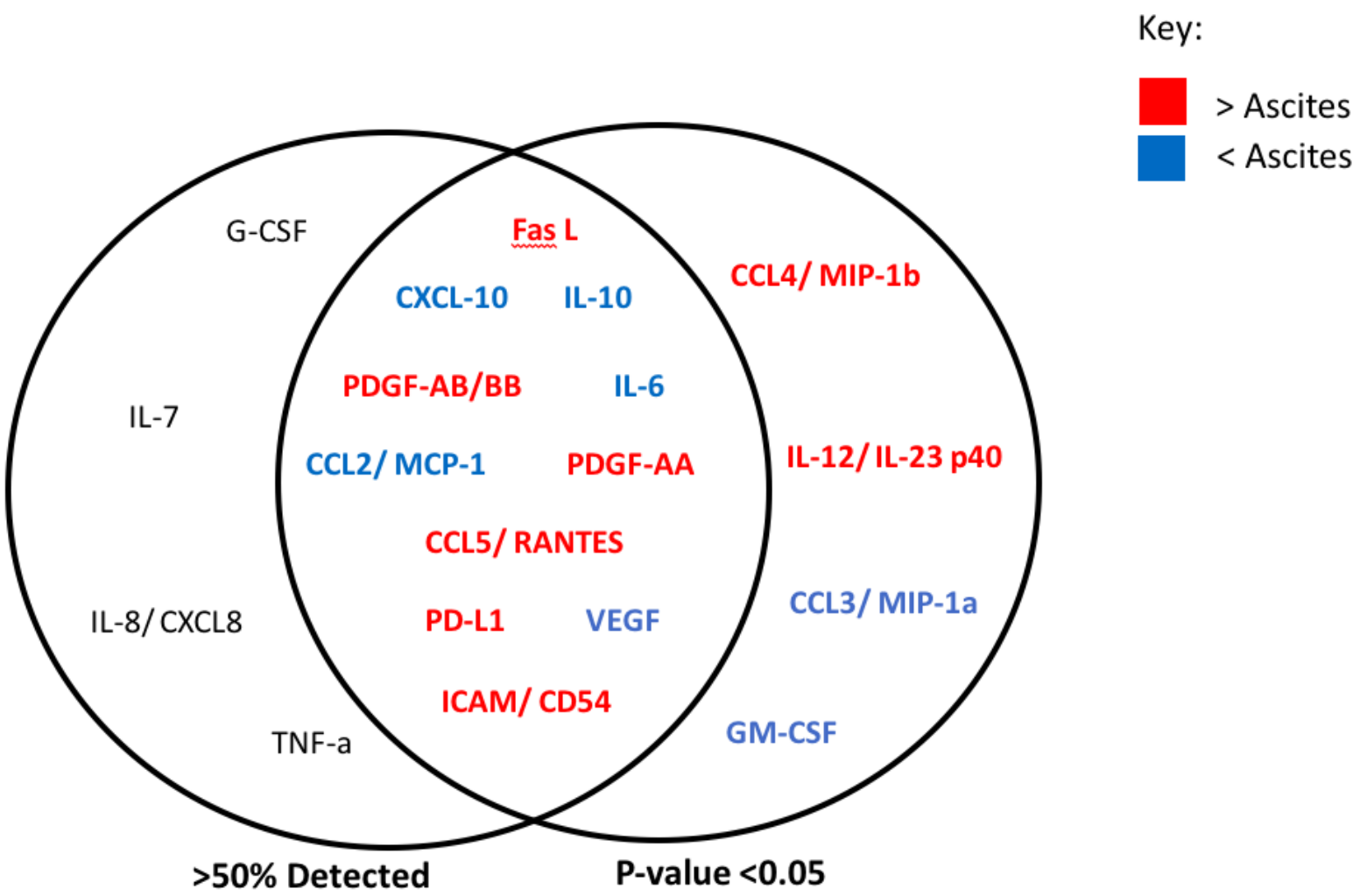

Table 9 Non-Significant and Undetected Analytes

Non-Significant and Undetected Analytes
CD40L
IL-1α
IL-1β
IL-17a
IFN-α
IFN-γ
IL-12 p70
VEGF
IL-4
IL-2
IL-5
TRAIL
TGF-α
TNF-β

Figure 19 Plasma and ascites were analysed by Luminex multiplex assays for 39 patients and 4 healthy donors
Graphical illustration of detected analyte concentrations which were significantly different between plasma and ascites.

In aims to reduce the impact of batch effect, concentrations were converted to log(2) Z score values for each analyte (Figure 20). Despite normalising the data, batch effect remained problematic, particularly across ascites samples.

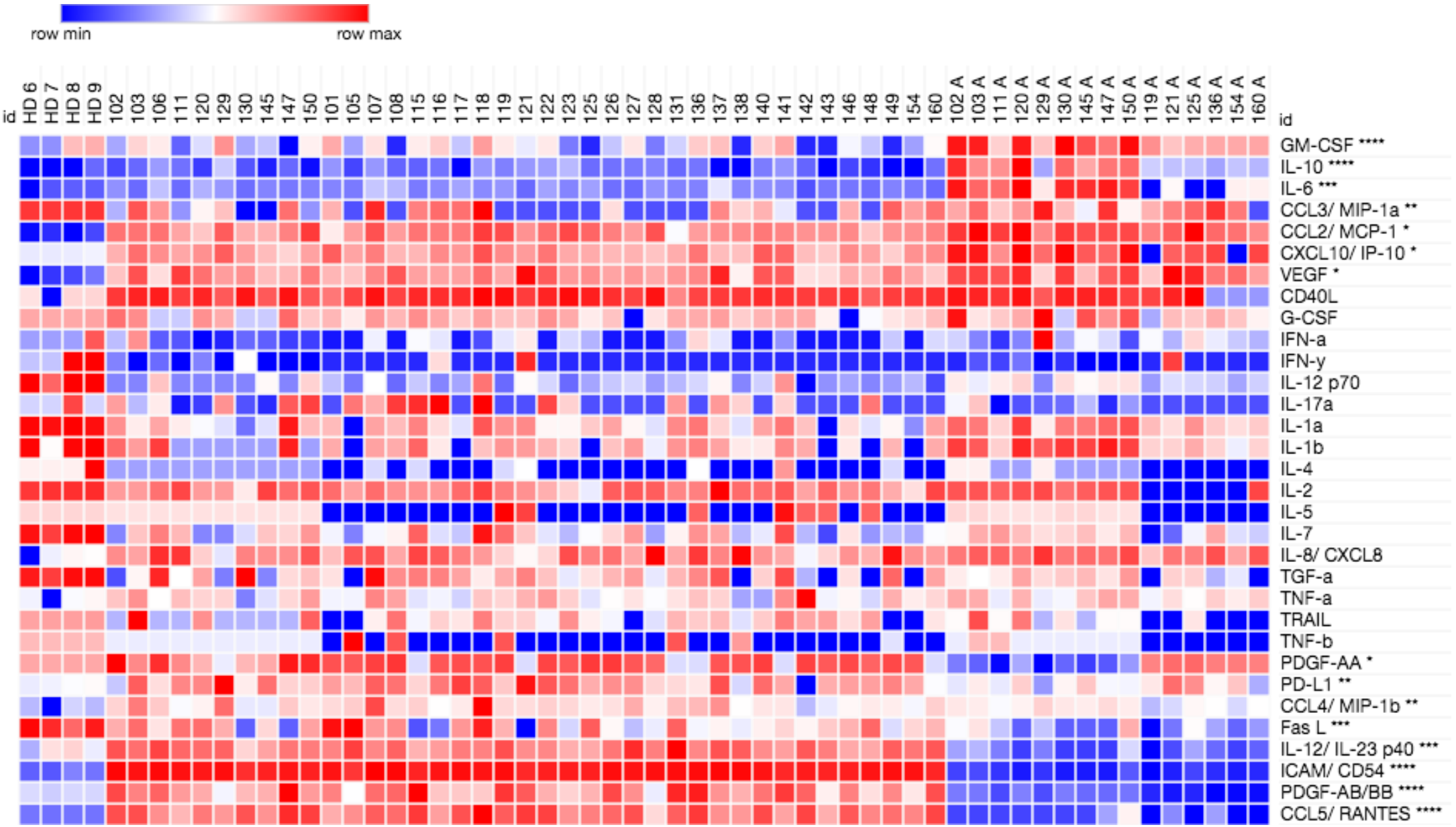

Figure 20 Plasma and ascites were analysed by Luminex multiplex assays for 39 patients and 4 healthy donors

Graphical illustration showing the differing concentrations of analytes between plasma and ascites. Data shown at Log(2) Z score of analyte concentrations. Asterisks represent statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

To conclude, a number of analytes were undetected by Luminex. IL-17A, IFN-γ, IL-12p70, IL-4, IL-5 and TNF-β require alternative immunoassays to determine accurate concentrations within plasma and ascites. CXCL10, IL-10, IL-6, CCL2, VEGF, Fas Ligand, PDGF-AB/BB, PDGF-AA, CCL5, PD-L1 and ICAM had significantly different concentrations between compartments and had a detection rate greater than 50%. Analysis was performed using 39 patient samples and 4 healthy donors, Luminex must be performed on all 115 samples in order to confirm trends within the cohort. Once this has been achieved, further investigation must be performed to establish the role of these analytes in the progression and severity of liver disease, and correlation with infection. Batch effect has proven a major issue in analysis whereby Luminex kit and date of experiment performed has resulted in disparities. Despite normalising data, batch effect remained an issue across patient samples.

6. Discussion

Liver cirrhosis is associated with systemic inflammation, which can progress to immune dysregulation in advanced cirrhosis. Immune characteristics and dysfunction may thus correlate with disease severity, which may inform novel diagnostic tests, patient stratification and targeted interventions. The aim of this project was to gain further insight into the immune profiles of individuals with liver cirrhosis and establish correlations with disease state and severity. Cellular and soluble immune profiles were investigated, using flow cytometry and Luminex, respectively, in order to further understand immune profiles of patients with varying stages and manifestations of liver cirrhosis.

Patients categorized with no acute illness, with CP-A and -B, demonstrated similar immune characteristics throughout this study. These patients had stable cirrhosis indicative of normal hepatic function, which was reflected by similar cellular proportions compared to healthy donors suggestive of a well-functioning immune system. Patients with CP-B differed however from healthy donors in the proportions of MAIT and $CD8^+$ T cells. The depletion of both lymphocyte subsets may play a role in the increased risk of mortality in patients with CP-B due to compromised immune surveillance against infection and malignancies.

Liver cirrhosis can progress from a compensated with or without portal hypertension and the development of varices to a decompensated phase, which is associated variceal bleeding, encephalopathy, ascites and risk of associated bacterial infections. Patients with chronic decompensation were characterised by an increased proportion $CD4^+$ T cells and depletion of $CD8^+$ T cells in blood. Conversely, when comparing immune profiles of blood versus ascites, the ascites had reduced $CD4^+$ T cells and an increased proportion of $CD8^+$ T cells. Thus, it can be suggested that blood $CD8^+$ T cells preferentially migrate towards the ascitic fluid. The ascitic $CD8^+$ T cells had pronounced expression of CD69 and PD1. An earlier study involving 60 cirrhotic patients also observed an upregulation of PD1 on $CD8^+$ T cells in the ascites compared to cells in peripheral blood (Lebossé et al., 2019). The expression of these markers and subsequent activation of $CD8^+$ T cells implies a role of this subset in local immune surveillance with ascites. PD-L1 and PD-L2 binds to PD-1 receptor expressed on T cells and exerts inhibitory

effects on T cell function. The upregulation of PD1 in parallel to low Ki67 expression may imply that $CD8^+$ T cells have entered an exhausted state (Jubel et al., 2020).

In contrast to chronic decompensation, acute decompensation is a result of the severe and sudden development of manifestations previously noted. Patients had reduced proportions of $CD8^+$ T cells and MAIT cells within the peripheral blood, and a greater percentage of NKT cells. Similarly to chronic decompensation, ascites exhibited a depletion of $CD4^+$ T cells.

No significant trends in cellular and soluble immune components were observed in patients with acute decompensated cirrhosis in the presence of infection. This lack of infection-specific signatures may be due to differences in the nature of the causative organisms and anatomical location of infections. Patients within this group presented with SBP and non-SBP infections, which included pneumonia, gastroenteritis and urinary tract infection. The range of infections in the study population may account for the lack of cellular differences and subsequent variation in immune response. In addition, the sample size of patients with diagnosed infection was limited. Recruiting a larger cohort of patients may draw out trends regarding immune responses to infections in cirrhotic patients.

ACLF is associated with severe systemic inflammation triggered by PAMPs-elicited innate immune cell activation (Clària et al., 2016 and Moreau et al., 2020). The poor prognosis and lack of reasoning as to why some patients develop ACLF demands further investigation in order to identify potential biomarkers and develop therapeutic solutions. Clinically, ACLF was associated with anaemia, leukocytosis, neutrophilia and monocytosis. Despite no variation in the absolute lymphocyte count, the proportion of $CD4^+$ T cells was increased compared to healthy controls. A study by Rueschenbaum et al. (2021) observed a reduction of naïve and effector $CD4^+$ T cells, but an increase in $CD4^+$ regulatory T cells amongst patients with ACLF. This may suggest that the increase of $CD4^+$ regulatory T cells could be responsible for the increased proportion of $CD4^+$ T cells noted in the present study. In accordance with those findings, circulating $CD8^+$ T cells were reduced in ACLF in the present study. In keeping with Rueschenbaum et al. (2021) ascites had elevated expression of PD1 in $CD4^+$ and $CD8^+$ T cell subset in ACLF. Depletion of $CD8^+$ T cells in parallel with marked expression of PD1 may be a consequence of immunosuppression in advanced liver disease, leading to increased incidence

of new and recurrent infection in ACLF. Blockade of PD1 receptor may reverse T cells back to a functioning state to eradicate opportunistic pathogens and therefore has potential as a novel therapeutic strategy for ACLF.

MAIT cells were diminished in patients with no acute illness (CP-B), acute decompensation and ACLF, in notion with Riva et al. (2017) and Zhang et al. (2021). MAIT cells are abundant in the liver with pro-inflammatory anti-microbial functions. Due to their role in immunosurveillance, the depletion of MAIT cells may predispose patients to infection. In particular, patients with acute decompensation had significantly lower proportion of MAIT cells compared to healthy donors, chronic decompensated patients and ACLF, suggesting that acutely decompensated patients may have an increased susceptibility to opportunistic microbial infection. Zhang et al. (2021) suggested the depletion of MAIT cells may be due to re-distribution, cell exhaustion, or induced cell death. In the present study, despite a lack of statistical significance, ascites had a greater proportion of MAIT cells than peripheral blood, suggesting that MAIT cells migrate from circulation into ascitic fluid. Zhang et al. (2021) also implied the reduction in MAIT cells may be due to cytokine-driven programmed cell death, thus further investigation into the cytokine profile of ACLF is needed to establish the cause of MAIT cell depletion.

An aim of the study was to identify potential biomarkers predictive of SBP. SBP was associated with a higher proportion of activated NK cells, in addition to an increase in $CD8^+$ T cells and reduction of $CD4^+$ T cells in blood. A previous study involving 8 patients with SBP infection also observed an upregulation of CD69 expression in ascites NK cells associated with SBP (Lutz et al., 2019), suggesting NK cells play a role in immunity against SBP. Despite this, Lutz et al. (2019) contrastingly noted a decrease in NK cell frequency in patients with SBP compared to patients without. An insufficient number of patients with detected SBP were recruited in both studies to yield statistical power concerning the immune profiles of SBP. A larger cohort is required to determine to phenotype conventional and non-conventional lymphocytes in SBP. Ascites $CD4^+$ and $CD8^+$ T cell populations also expressed elevated levels of PD1 implying T cell exhaustion, thus further investigation is required to verify the functional state of T cells within the ascitic fluid. Exhaustive T cells may therefore be key in the immunosuppression and subsequent high-risk mortality related to SBP. Moreover, CD69 expression was diminished in

ascites of $CD4^+$ and $CD8^+$ T cells in the one patient with ACLF concurrent with SBP, despite being upregulated in acute decompensated SBP patients. This may further support the notion of T cell exhaustion and immune dysfunction in ACLF, particularly in the presence of SBP.

The absolute number of peripheral blood monocytes was significantly increased with increasing severity of liver cirrhosis. This suggests that monocytes may play a key role in immune dysregulation in liver disease. Further investigation is required to establish key monocytic phenotypes involved in cirrhosis.

Batch effect limited analysis of humoral profiles in cirrhosis, however trends were analysed between compartments of paired samples. IL-6, CXCL10, VEGF, CCL5, Fas ligand, soluble PD-L1 (sPD-L1), PDGF-AB/BB, PDGF-AA and soluble ICAM-1 (sICAM-1) were enriched in the ascitic fluid. In notion with this study, Lutz et al. (2019) observed elevated levels of CXCL10 in the ascites. One function of CXCL10 is the chemoattraction of NK cells, which corresponds with the increased NK population observed here in ascites of SBP patients. VEGF levels were also elevated in ascites. Triggered by inflammation and hypoxia, VEGF promotes angiogenesis, fibrogenesis and increases vascular permeability. The hypoxic state of ascites coupled with inflammation may suggest VEGF is produced locally in ascites. Abdelmoaty et al. (2009) found elevated serum VEGF levels related to liver cirrhosis, with a positive association with CP score. Additionally, several studies have shown a link between high serum VEGF concentration and development of HCC (Li et al., 2004, Zhang et al., 2014, and Mukozu et al., 2012). Analysis of plasma VEGF levels in progressive liver disease would be required to confirm this. Ascites CCL5 and Fas levels were higher than in blood. CCL5 is a chemoattractant for monocytes, lymphocytes and myeloid cells, namely basophils and eosinophils. This suggests that CCL5 may be responsible for the migration of some immune cells into the ascitic fluid. Kurys-Denis et al. (2020) observed a positive correlation between plasma PDGF-AB/BB and PDGF-AA and severity of alcoholic liver cirrhosis. In the current study, analyte levels were compared exclusively between compartments. Analysis of these analytes in peripheral blood across groups is needed to confirm their role in disease severity. Similarly to PD-1 expression analysed by flow cytometry, sPD-L1 was also increased in the ascites. Again, this may be suggestive of T cell dysfunction and exhaustion contributing to immunosuppression and increased susceptibility to infection.

Analysis of analytes levels in the plasma alone is required to identify correlations with disease progression and severity. To do this, the Luminex experiments should be re-run to reduce batch effect.

6.1 Limitations and Future Work

My study had several limitations. COVID restrictions prevented the recruitment of patients from the beginning of the pandemic in 2020 until April 2021. This restricted the number of participants and experiments conducted for this project. As a result, only four patients with SBP were recruited onto the study resulting in the inability to establish markers distinctive of SBP. In principle, a larger cohort of patients with detected SBP would be required to identify immune characteristics. Moreover, clinical data reveal that monocytes may play a key role in cirrhosis-related immunosuppression; thus, analysis of monocyte phenotypes should be a research priority.

Magnetic Luminex performance assays were carried out on two separate occasions. As a consequence, batch effect presented an unexpected problem whereby the different variables i.e. day of experiment and batch of Luminex plate led to considerable differences in concentration values produced by the machine and thus analysed. However, this project was primarily looking at trends observed amongst analytes in plasma and ascites patient samples. Therefore, the batch effect had a lesser impact on these analyses. To eliminate batch effect in the future, Luminex assays should be carried out on a single day with same plate batch if feasible to reduce variability. This would be particularly important when comparing analyte concentrations across patient groups. Furthermore, as Luminex is useful to identify trends, ELISAs could be carried out on analytes which show promising results in order to produce more accurate concentrations and findings of these analytes of interest. Another method to enhance analysis would be to incorporate clustering tools to unbiasedly stratify patients and establish immune patterns currently unidentified.

Future objectives:

- Undertake a large-scale Luminex multiplex assay involving all patient samples
- Undertake functional T cell assays to determine their activation
- Complete analysis of MDSC flow cytometry
- Integrate all cellular, soluble and clinical data
- Incorporate non-directed clustering tools for unbiased stratification of patients and establish immune signatures of progressive liver cirrhosis

7. Conclusions

To conclude, MAIT cells were significantly reduced in patients with no acute illness, acute decompensation and ACLF. Due to their anti-microbial role, the depletion of MAIT cells may predispose patients to infection. Overexpression of PD1, and subsequent T cell exhaustion, may also impair the cytotoxic function of T cells leading to an increased susceptibility to opportunistic pathogens. Moreover, my study suggests that alterations in the monocytic lineage across patient groups may correlate with disease severity and act as an additional mechanism for immunosuppression. Further investigation of monocytic phenotypes and frequencies is required to identify trends between patient groups. To reduce batch effect in Luminex analysis, all samples should be analysed simultaneously in the foreseeable future to validate the importance of analytes of interest in liver cirrhosis.

8. References:

Abdelmoaty, M., Bogdady, A., Attia, M. and Zaky, N., 2009. Circulating vascular endothelial growth factor and nitric oxide in patients with liver cirrhosis: A possible association with liver function impairment. *Indian Journal of Clinical Biochemistry*, 24(4), pp.398-403.

Albillos, A., Lario, M. and Álvarez-Mon, M., 2014. Cirrhosis-associated immune dysfunction: Distinctive features and clinical relevance. *Journal of Hepatology*, 61(6), pp.1385-1396.

Arroyo, V., Moreau, R. and Jalan, R., 2020. Acute-on-Chronic Liver Failure. *New England Journal of Medicine*, 382(22), pp.2137-2145.

Asrani, S., Devarbhavi, H., Eaton, J. and Kamath, P., 2019. Burden of liver diseases in the world. *Journal of Hepatology*, 70(1), pp.151-171.

British Liver Trust. 2019. *Statistics - British Liver Trust*. [online] Available at: <https://britishlivertrust.org.uk/about-us/media-centre/statistics/> [Accessed 13 March 2021].

Bucsics, T. and Krones, E., 2017. Renal dysfunction in cirrhosis: acute kidney injury and the hepatorenal syndrome. *Gastroenterology Report*, 5(2), pp.127-137.

Carbonell, N., Pauwels, A., Serfaty, L., Fourdan, O., Lévy, V. and Poupon, R., 2004. Improved survival after variceal bleeding in patients with cirrhosis over the past two decades. *Hepatology*, 40(3), pp.652-659.

Cárdenas, A. and Ginès, P., 2005. Management of Refractory Ascites. *Clinical Gastroenterology and Hepatology*, 3(12), pp.1187-1191.

Clària, J., Stauber, R., Coenraad, M., Moreau, R., Jalan, R., Pavesi, M., Amorós, À., Titos, E., Alcaraz-Quiles, J., Oettl, K., Morales-Ruiz, M., Angeli, P., Domenicali, M., Alessandria, C., Gerbes, A., Wendon, J., Nevens, F., Trebicka, J., Laleman, W., Saliba, F., Welzel, T., Albillos, A., Gustot, T., Benten, D., Durand, F., Ginès, P., Bernardi, M. and Arroyo, V., 2016. Systemic

inflammation in decompensated cirrhosis: Characterization and role in acute-on-chronic liver failure. *Hepatology*, 64(4), pp.1249-1264.

D'Amico, G., Garcia-Tsao, G. and Pagliaro, L., 2006. Natural history and prognostic indicators of survival in cirrhosis: A systematic review of 118 studies. *Journal of Hepatology*, 44, pp.217-231.

D'Amico, G., Pasta, L., Morabito, A., D'Amico, M., Caltagirone, M., Malizia, G., Tinè, F., Giannuoli, G., Traina, M., Vizzini, G., Politi, F., Luca, A., Virdone, R., Licata, A. and Pagliaro, L., 2014. Competing risks and prognostic stages of cirrhosis: a 25-year inception cohort study of 494 patients. *Alimentary Pharmacology & Therapeutics*, 39(10), pp.1180-1193.

El-Serag, H., 2012. Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. *Gastroenterology*, 142(6), pp.1264-1273.e1.

Feather, A., Randall, D., Waterhouse, M. and Kumar, P., 2020. *Kumar & Clark's clinical medicine*. 10th ed. Elsevier, pp.1261-1312.

Fernández, J., Monteagudo, J., Bargallo, X., Jiménez, W., Bosch, J., Arroyo, V. and Navasa, M., 2005. A randomized unblinded pilot study comparing albumin versus hydroxyethyl starch in spontaneous bacterial peritonitis. *Hepatology*, 42(3), pp.627-634.

Garcia-Tsao, G. and Bosch, J., 2015. Varices and Variceal Hemorrhage in Cirrhosis: A New View of an Old Problem. *Clinical Gastroenterology and Hepatology*, 13(12), pp.2109-2117.

Garrison, R., Cryer, H., Polk, H., and Howard, D., 1984. Clarification of Risk Factors for Abdominal Operations in Patients with Hepatic Cirrhosis. *Annals of Surgery*, 199(6), pp.648-655.

Godfrey, D., Uldrich, A., McCluskey, J., Rossjohn, J. and Moody, D., 2015. The burgeoning family of unconventional T cells. *Nature Immunology*, 16(11), pp.1114-1123.

Gustot, T., 2011. Multiple organ failure in sepsis: prognosis and role of systemic inflammatory response. *Current Opinion in Critical Care*, 17(2), pp.153-159.

Gustot, T., Fernandez, J., Garcia, E., Morando, F., Caraceni, P., Alessandria, C., Laleman, W., Trebicka, J., Elkrief, L., Hopf, C., Solís-Munoz, P., Saliba, F., Zeuzem, S., Albillos, A., Benten, D., Montero-Alvarez, J., Chivas, M., Concepción, M., Córdoba, J., McCormick, A., Stauber, R., Vogel, W., de Gottardi, A., Welzel, T., Domenicali, M., Risso, A., Wendon, J., Deulofeu, C., Angeli, P., Durand, F., Pavesi, M., Gerbes, A., Jalan, R., Moreau, R., Ginés, P., Bernardi, M. and Arroyo, V., 2015. Clinical Course of acute-on-chronic liver failure syndrome and effects on prognosis. *Hepatology*, 62(1), pp.243-252.

Iwakiri, Y., 2014. Pathophysiology of Portal Hypertension. *Clinics in Liver Disease*, 18(2), pp.281-291.

Jalan, R. and Williams, R., 2002. Acute-on-Chronic Liver Failure: Pathophysiological Basis of Therapeutic Options. *Blood Purification*, 20(3), pp.252-261.

Jubel, J., Barbati, Z., Burger, C., Wirtz, D. and Schildberg, F., 2020. The Role of PD-1 in Acute and Chronic Infection. *Frontiers in Immunology*, 11.

Karagozian, R., Rutherford, A., Christopher, K. and Brown J, R., 2016. Spontaneous bacterial peritonitis is a risk factor for renal failure requiring dialysis in waitlisted liver transplant candidates. *Clinical Transplantation*, 30(5).

Kurys-Denis, E., Prystupa, A., Luchowska-Kocot, D., Krupski, W., Bis-Wencel, H. and Panasiuk, L., 2020. PDGF-BB homodimer serum level – a good indicator of the severity of alcoholic liver cirrhosis. *Annals of Agricultural and Environmental Medicine*, 27(1), pp.80-85.

Lebossé, F., Gudd, C., Tunc, E., Singanayagam, A., Nathwani, R., Triantafyllou, E., Pop, O., Kumar, N., Mukherjee, S., Hou, T., Quaglia, A., Zoulim, F., Wendon, J., Dhar, A., Thursz, M., Antoniades, C. and Khamri, W., 2019. CD8+ T cells from patients with cirrhosis display a

phenotype that may contribute to cirrhosis-associated immune dysfunction. *EBioMedicine*, 49, pp.258-268.

Li, X., 2004. Expression of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma and effect of transcatheter arterial chemoembolization therapy on plasma vascular endothelial growth factor level. *World Journal of Gastroenterology*, 10(19), p.2878.

Louten, J., Boniface, K. and de Waal Malefyt, R., 2009. Development and function of TH17 cells in health and disease. *Journal of Allergy and Clinical Immunology*, 123(5), pp.1004-1011.

Lutz, P., Jeffery, H., Jones, N., Birtwistle, J., Kramer, B., Nattermann, J., Spengler, U., Strassburg, C., Adams, D. and Oo, Y., 2019. NK Cells in Ascites From Liver Disease Patients Display a Particular Phenotype and Take Part in Antibacterial Immune Response. *Frontiers in Immunology*, 10.

Mansour, A., Watson, W., Shayani, V. and Pickleman, J., 1997. Abdominal operations in patients with cirrhosis: Still a major surgical challenge. *Surgery*, 122(4), pp.730-736.

Meng, F., Wang, K., Aoyama, T., Grivennikov, S., Paik, Y., Scholten, D., Cong, M., Iwaisako, K., Liu, X., Zhang, M., Österreicher, C., Stickel, F., Ley, K., Brenner, D. and Kisseleva, T., 2012. Interleukin-17 Signaling in Inflammatory, Kupffer Cells, and Hepatic Stellate Cells Exacerbates Liver Fibrosis in Mice. *Gastroenterology*, 143(3), pp.765-776.e3.

Moreau, R., Clària, J., Aguilar, F., Fenaille, F., Lozano, J., Junot, C., Colsch, B., Caraceni, P., Trebicka, J., Pavesi, M., Alessandria, C., Nevens, F., Saliba, F., Welzel, T., Albillos, A., Gustot, T., Fernández, J., Moreno, C., Baldassarre, M., Zaccherini, G., Piano, S., Montagnese, S., Vargas, V., Genescà, J., Solà, E., Bernal, W., Butin, N., Hautbergue, T., Cholet, S., Castelli, F., Jansen, C., Steib, C., Campion, D., Mookerjee, R., Rodríguez-Gandía, M., Soriano, G., Durand, F., Benten, D., Bañares, R., Stauber, R., Gronbaek, H., Coenraad, M., Ginès, P., Gerbes, A., Jalan, R., Bernardi, M., Arroyo, V. and Angeli, P., 2020. Blood metabolomics uncovers inflammation-

associated mitochondrial dysfunction as a potential mechanism underlying ACLF. *Journal of Hepatology*, 72(4), pp.688-701.

Moreau, R., Jalan, R., Gines, P., Pavesi, M., Angeli, P., Cordoba, J., Durand, F., Gustot, T., Saliba, F., Domenicali, M., Gerbes, A., Wendon, J., Alessandria, C., Laleman, W., Zeuzem, S., Trebicka, J., Bernardi, M. and Arroyo, V., 2013. Acute-on-Chronic Liver Failure Is a Distinct Syndrome That Develops in Patients With Acute Decompensation of Cirrhosis. *Gastroenterology*, 144(7), pp.1426-1437.e9.

Mosser, D. and Edwards, J., 2010. Erratum: Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*, 10(6), pp.460-460.

Mukozu, T., Nagai, H., Kobayashi, K., Ogino, Y., Matui, D., Kanekawa, T., Kogame, M., Takayama, R., Kanayama, M., Wakui, N., Shinohara, M., Shinohara, M., Ishii, K., Igarashi, Y. and Sumino, Y., 2012. Su2042 Serum VEGF as a Tumor Marker for Patients With HCV-Related Liver Cirrhosis and Hepatocellular Carcinoma. *Gastroenterology*, 142(5), p.S-980.

Murphy, K. and Weaver, C., 2016. *Janeway's Immunobiology*. 9th ed. Taylor and Francis, pp.1-25.

Nicholson, L., 2016. The immune system. *Essays in Biochemistry*, 60(3), pp.275-301.

Ono, Y., Watanabe, T., Matsumoto, K., Ito, T., Kunii, O. and Goldstein, E., 2004. Opsonophagocytic dysfunction in patients with liver cirrhosis and low responses to tumor necrosis factor-α and lipopolysaccharide in patients' blood. *Journal of Infection and Chemotherapy*, 10(4), pp.200-207.

Pedersen, J., Bendtsen, F. and Møller, S., 2015. Management of cirrhotic ascites. *Therapeutic Advances in Chronic Disease*, 6(3), pp.124-137.

Pellicci, D., Koay, H. and Berzins, S., 2020. Thymic development of unconventional T cells: how NKT cells, MAIT cells and γδ T cells emerge. *Nature Reviews Immunology*, 20(12), pp.756-770.

Pimpin, L., Cortez-Pinto, H., Negro, et al. 2018. Burden of liver disease in Europe: Epidemiology and analysis of risk factors to identify prevention policies. *Journal of Hepatology*, 69(3), pp.718-735.

Planas, R., Montoliu, S., Ballesté, B., Rivera, M., Miquel, M., Masnou, H., Galeras, J., Giménez, M., Santos, J., Cirera, I., Morillas, R., Coll, S. and Solà, R., 2006. Natural History of Patients Hospitalized for Management of Cirrhotic Ascites. *Clinical Gastroenterology and Hepatology*, 4(11), pp.1385-1394.e1.

Riva, A., Patel, V., Kurioka, A., Jeffery, H., Wright, G., Tarff, S., Shawcross, D., Ryan, J., Evans, A., Azarian, S., Bajaj, J., Fagan, A., Patel, V., Mehta, K., Lopez, C., Simonova, M., Katzarov, K., Hadzhiolova, T., Pavlova, S., Wendon, J., Oo, Y., Klenerman, P., Williams, R. and Chokshi, S., 2017. Mucosa-associated invariant T cells link intestinal immunity with antibacterial immune defects in alcoholic liver disease. *Gut*, 67(5), pp.918-930.

Rueschenbaum, S., Ciesek, S., Queck, A., Widera, M., Schwarzkopf, K., Brüne, B., Welsch, C., Wedemeyer, H., Zeuzem, S., Weigert, A. and Lange, C., 2021. Dysregulated Adaptive Immunity Is an Early Event in Liver Cirrhosis Preceding Acute-on-Chronic Liver Failure. *Frontiers in Immunology*, 11.

Sort, P., Navasa, M., Arroyo, V., Aldeguer, X., Planas, R., Ruiz-del-Arbol, L., Castells, L., Vargas, V., Soriano, G., Guevara, M., Ginès, P. and Rodés, J., 1999. Effect of Intravenous Albumin on Renal Impairment and Mortality in Patients with Cirrhosis and Spontaneous Bacterial Peritonitis. *New England Journal of Medicine*, 341(6), pp.403-409.

Tapper, E., Henderson, J., Parikh, N., Ioannou, G. and Lok, A., 2019. Incidence of and Risk Factors for Hepatic Encephalopathy in a Population-Based Cohort of Americans With Cirrhosis. *Hepatology Communications*, 3(11), pp.1510-1519.

Thévenot, T., Bureau, C., Oberti, et al. 2014. Effect of albumin in cirrhotic patients with infection other than spontaneous bacterial peritonitis. A randomized trial. *Journal of Hepatology*, 62(4), pp.822-30.

Tokita, D., Sumpter, T., Raimondi, G., Zahorchak, A., Wang, Z., Nakao, A., Mazariegos, G., Abe, M. and Thomson, A., 2008. Poor allostimulatory function of liver plasmacytoid DC is associated with pro-apoptotic activity, dependent on regulatory T cells. *Journal of Hepatology*, 49(6), pp.1008-1018.

Tsoris, A. and Marlar, C., 2022. Use Of The Child Pugh Score In Liver Disease [online] Ncbi.nlm.nih,gov. Available at: ,https://www.ncbi.nlm.nih.gov/books/NBK542308/> [Accessed 18 May 2022]

Vilstrup, H., Amodio, P., Bajaj, J., Cordoba, J., Ferenci, P., Mullen, K., Weissenborn, K. and Wong, P., 2014. Hepatic encephalopathy in chronic liver disease: 2014 Practice Guideline by the American Association for the Study Of Liver Diseases and the European Association for the Study of the Liver. *Hepatology*, 60(2), pp.715-735.

Wong, Y., Qiu, T., Tam, Y., Mohan, B., Gallegos-Orozco, J., and Adler, D., 2020.Efficacy and Safety of IV albumin for non-spontaneous bacterial peritonitis infection among patients with cirrhosis: A systematic review and meta-analysis. *Digestive and Liver Disease*, 52(10), pp.1137-1142.

Wang, M. and Ju, C., 2017. Role of macrophages in alcoholic liver disease. *Alcohol*, 59, p.76.

Williams, R., 2015. Liver disease in the UK: Startling findings & urgent need for action. *Journal of Hepatology*, 63(2), pp.297-9.

Zhang, Y., Fan, Y., He, W., Han, Y., Bao, H., Yang, R., Wang, B., Kong, D. and Wang, H., 2021. Persistent deficiency of mucosa-associated invariant T (MAIT) cells during alcohol-related liver disease. *Cell & Bioscience*, 11(1).

Zhang, W., Kim, R., Quintini, C., Hashimoto, K., Fujiki, M., Diago, T., Eghtesad, B., Miller, C., Fung, J., Tan, A., Menon, K. and Aucejo, F., 2014. Prognostic role of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma undergoing liver transplantation. *Liver Transplantation*, 21(1), pp.101-111.

Zheng, M. and Tian, Z., 2019. Liver-Mediated Adaptive Immune Tolerance. *Frontiers in Immunology*, 10.